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MATRIX METALLOPROTEINASES: ACTIVITY AND INHIBITION

BY

MALTI PANDURANG NIKRAD

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**.

IN

ORAL BIOLOGY

FACULTY OF DENTISTRY

EDMONTON, ALBERTA

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DATED. April 30 .,1993

To

Pandurang, Mrinalini, Himgauri

and

My Parents

ABSTRACT

Matrix Metalloproteinases (MMPs) are responsible for the physiological and pathological destruction of connective tissue proteins which include the different types of collagens, proteoglycans and glycoproteins. Collagenase (MMP-1) is known to be specific for cleaving triple helical collagen. Several lines of evidence suggest that the intermolecular cross-links in fibrillar collagen make it less susceptible to the action of collagenase. Lysosomal enzymes (that have an acidic pH optimum) and polymorphonuclear leukocyte elastase (released during inflamation) are effective in solubilizing fibrillar collagen, but these may not be responsible for degrading extracellular fibrillar collagen under physiological conditions. Thus a role was suggested for an enzyme that was active against the C-telopeptide of collagen, in the extracellular matrix, with a neutral pH optimum. Previous work in this department has shown the presence of an enzyme in porcine and human gingival explants active at neutral pH against collagen. The enzyme thought to be responsible for this activity was called "telopeptidase" (MMP-4).

Telopeptidase was purified from human gingival fibroblast culture medium. The medium was concentrated, activated and applied to a gel filtration column. The fractions containing telopeptidase activity (tested against acid soluble fetal bovine skin collagen reduced with NaB^3H_4 which labels the lysine involved in the cross-links) were pooled and further separated by chromatofocusing. Collagenase was obtained in the purified form after this step. Telopeptidase eluted as two separated peaks on chromatofocusing and these were further purified separately by hydrophobic interaction chromatography with ammonium sulphate. Both

telopeptidase and gelatinase (MMP-2) eluted together after 50% ethylene glycol was applied. Hydrophobic interaction chromatography was repeated on the two separated fractions without adding ammonium sulphate. Telopeptidase did not bind to Phenyl-Sepharose whereas gelatinase eluted with 50% ethylene glycol.

To define the peptide bond in the C-telopeptide of type I collagen that was cleaved by telopeptidase, two peptides were synthesized. One peptide included the N-terminal 12 amino acids (which included three helical amino acids) of the C-telopeptide of the $\alpha_1$chain of type I collagen and the second contained the C-terminal 15 amino acids. The sites on these peptide substrates cleaved by purified telopeptidase, purified and recombinant stromelysin (MMP-3) and purified and recombinant collagenase were determined.

If, as proposed, telopeptidase is responsible for the initial solubilization of fibrillar collagen followed by the action of collagenase and then the other activities, a specific inhibitor to telopeptidase when used in fibroblast cell culture (where other matrix metalloproteinases are secreted by fibroblasts), should inhibit collagen breakdown. With this in mind, peptide analogs of the C-telopeptide sequence N-terminal to the telopeptidase cleavage site were synthesized. These peptides were converted into C-terminal hydroxamates. Di-, tri-, and tetrapeptides containing different amino acids were synthesized and tested against the three enzymes. The best inhibitor in the series was Z-(L)-Pro-Pro-Leu-Gly-NHOH inhibited with an IC_{50} of 29 μM for MMP-1, 1.61 μM for MMP-2 and 3.8 μM for MMP-3. Some of these peptide inhibitors were shown to inhibit the destruction of fluorescently-labelled fibrillar collagen by skin and gingival fibroblasts *in vitro*.

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ABBREVATIONS

α_1C-telo-1	N-Acetyl-GPPSGGYDLSFLPQPPQEKAHDGGRYY-NH_2
α_1C-telo-2	N-Acetyl-PQPPQEKAHDGGRYY-NH_2
α_2-M	$Alpha_2$-Macroglobulin
ASC	Acid Soluble Collagen
CH_3CN	Acetonitrile
CNBr	Cyanogen bromide
DCC	N,N'-Dicyclohexylcarbodiimide
DMSO	Dimethylsulphoxide
dpm	disintegrations per minute
FITC	Fluorescein isothiocynate
Fmoc	9-Fluorenyl methoxycarbonyl
HFBA	Heptafluorobutyric acid
HOBt	N-Hydroxybenzotriazole
MMP-1	Matrix Metalloproteinase-1 / Collagenase
MMP-2	Matrix Metalloproteinase-2 / Gelatinase
MMP-3	Matrix Metalloproteinase-3 / Stromelysin
MMP-4	Matrix Metalloproteinase-4 / Telopeptidase
M_r	Relative molecular weight
Nα_1C-telo	N-Acetyl-GPPSGGYDLSFL-NH_2
PDS	Proteodermatan sulphate
RP-HPLC	Reversed Phase High Performance Liquid SDS Sodium dodecyl

	sulphate
TEMED	N,N,N',N'-Tetramethylethylenediamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TIMP	Tissue Inhibitor of Metalloproteinase
TLC	Thin Layer Chromatography
Tris	Tris(hydroxymethyl) aminomethane
Z	Benzyloxycarbonyl

Amino acid symbols used in this manuscript

Amino acid symbol	Three-letter symbol	One-letter symbol
Alanine	Ala	A
Arginine	Arg	R
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Tyrosine	Tyr	Y

I. INTRODUCTION

A. The Extracellular Matrix

Connective tissue consists of an indigenous population of cells including fibroblasts or other cells of mesenchymal origin, surrounded by an intercellular matrix which they secrete. This matrix consists of fibrous proteins: collagens, elastin and ground substance: proteoglycans and glycoproteins. Collagen, the most ubiquitous of the proteins, comprises one third of the total body protein and is distributed throughout the connective tissues, i.e., in skin, ligaments, tendons, cartilage and matrix of mineralized tissue such as bone and dentin.

B. Collagen

B.1. Collagen Types

At least fourteen genetically different collagen types are associated with various connective tissues of the body. They have been classified by Van der Rest and Garrone, (1991) depending on their assembly. (1) Fibrillar collagens where the collagens participate in quarter-staggered fibrils. Types I, II, III, V, XI belong to this group. (2) Fibril associated collagens with interrupted triple helices (FACITs). The nonfibrillar collagen types IX, XII & XIV collagens belong to this group. (3) Collagens forming sheets. They are basement membrane type IV collagen and type VIII collagens. (4) Collagen forming beaded filaments. This class includes the type VI collagen.

B.2. Chemistry and Structure of Type I Collagen

Type I collagen is the major collagen component of all the interstitial connective tissues of mesenchymal origin. Other collagen types found in connective tissues, are designated type II to XIV. The interstitial collagen types I, II and III have several common features. The type I collagen molecule is constructed from three polypeptide chains, two of which are identical, and can be designated as: $[\alpha_1(I)]_2$ $\alpha_2(I)$. Different α chains are transcribed from different collagen genes. Types II and III collagens are composed of three identical chains denoted by $[\alpha_1(II)]_3$ and $[\alpha_1(III)]_3$ respectively. A single collagen molecule of these types is about 15A° in diameter and 3000°A in length. One third of the amino acids of collagen are glycine, while the imino acids, proline and hydroxyproline constitute one fourth of the total. Glycine is distributed throughout each of the α chains at every third residue in sequences of Gly-X-Y (Bornstein and Traub, 1979). The presence of glycine in every third position enables three collagen chains to associate to form the triple helices. Each polypeptide chain twists to the left, forming a left-handed helix. Three alpha chains are coiled around each other in a right-handed twist with a pitch of about 104°A to form a three-stranded super helix of the collagen molecule. In this conformation, the collagen molecule possesses a high degree of structural integrity, as well as resistance to the majority of non-specific proteinases. While the amino- and the carboxy- terminal extensions of the α-chains do not have glycine containing tripeptide sequences, they have a high number of acidic and basic amino acids. These regions of the molecule referred

to as the telopeptides, do not form triple helices and are susceptible to the action of certain proteinases (Bornstein and Sage, 1980).

B.3. Biosynthesis of Type I Collagen

The biosynthesis and assembly of the collagen monomer is a complex process and varies somewhat depending on the collagen types. As with other secretory proteins, the process of collagen synthesis begins in the nucleus of mesenchymal cells with the transcription of collagen genes, the α_1 (I) and α_2 (I) genes (reviewed by Prockop, 1982). Collagen genes appear to be about ten times longer than the corresponding mature mRNAs, with the coding information distributed in more than 54 short coding regions (exons) which are interrupted by noncoding regions (introns) of various sizes. The resulting mRNA is translated on membrane-bound ribosomes of the rough endoplasmic reticulum (Nimni, 1983).

The initially synthesized polypeptides (pre-proα chains) have N-terminal hydrophobic signal sequences which are removed during or shortly after translocation across the membrane of the rough endoplasmic reticulum (Nimni, 1983). Several posttranslational modifications which contribute to the stability of the collagen molecule and fibril then occur. These modifications include

(1) the formation of hydroxyproline and hydroxylysine,

(2) the addition of galactose and glucose residues to hydroxylysine.

The hydroxylation of peptidyl proline and lysine by prolyl and lysyl hydroxylases on pro-alpha chains takes place largely while the polypeptide chains are still being

assembled on the ribosomes in the cisternae of the rough endoplasmic reticulum (Kivirikko and Myllyla, 1979). Hydroxylation of proline on the collagen molecule is important for maintaining the helical conformation and thermal stability of the molecule. Hydroxylation of lysine contributes to collagen maturation and stability by serving as a cross-link component between collagen monomers in the fibrils. Triple helix formation occurs in the endoplasmic reticulum. After the addition of oligosaccharides (containing N-acetyl glucosamine and mannose) to the extension peptides, which occurs in the Golgi apparatus, the procollagen is packaged into secretory vesicles, transported to the plasma membrane and secreted from the cells (Nimni, 1983).

The propeptides are believed to prevent premature formation of collagen fibrils (Light and Bailey, 1980). Most of the pro-peptides are removed in the extracellular matrix by the respective N and C-terminal pro-peptidases after which the collagen monomers are incorporated into new or pre-existing collagen fibrils or fibers. The remaining segments of the extension peptides, which are referred to as the telopeptides (see Fig. 1A), are apparently essential for the proper alignment of the collagen molecules. Gelman et al. (1979) demonstrated that collagen molecules when treated with pepsin in order to remove most of the telopeptides, formed abnormal fibrils lacking the characteristic banding patterns. In addition the rate of fibril formation was significantly lower than with normal collagen (Brennan and Davison 1981). Some of the lysyl and hydroxylysyl residues on both the telopeptide regions of collagen are converted to aldehydes by the action of lysyl

Figure 1. Structure of Bovine Type I Collagen

A) Type I bovine skin collagen is composed of two α_1 and one α_2 chains. Each chain consists of 1,014 amino acid residues which make up the helical regions (represented by the horizontal line), and an additional 16 and 25 residues for the $\alpha_1$chain, located at the N and C-terminal extra-helical regions (telopeptides) respectively, and 9 and 6 residues for the α_2 chains respectively. The locations of the methionine residues are denoted by the short vertical lines.

a - denotes the peptides (CBX) derived from the treatment of the collagen with cyanogen bromide.

b - location of the cross-link precursor (N-9) in the N-terminal telopeptide.

c - location of the cross-link precursor (C-17) in the C-terminal telopeptide.

d - location of the bond cleaved by vertebrate collagenase (between residues 775-776).

B) A two dimensional representation of fibrillar collagen, showing the 4D stagger and intermolecular cross-linking between collagen molecule (see text).

b - residue N-9

c - residue-17

e - residue 87

f - residue 930

Intermolecular cross-links shown are between residues N-9 and 930, and residues C-17 and 87.

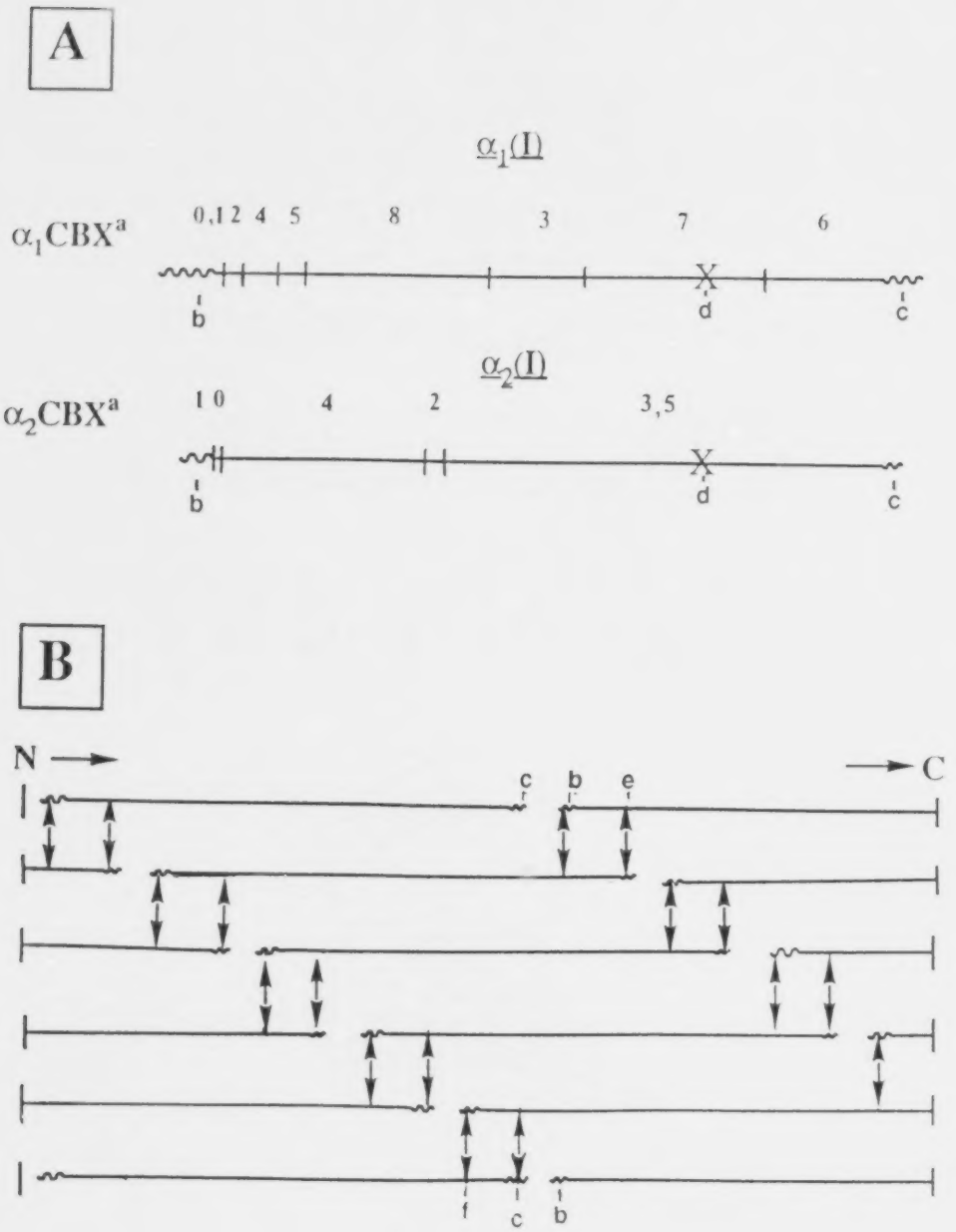


Fig. 1

oxidase and then form intra- and intermolecular cross-links.

B.4. Covalent Interchain Cross-links in Collagen

Various types of cross-links occur in collagen. The insolubility of a particular tissue is due to both the extent of cross-linking and the type of cross-link present. The cross-linking process can be prevented experimentally by inhibiting lysyl oxidase activity with beta-aminopropionitrile. When animals are fed sweet peas containing this chemical, the disease, osteolathyrism, is experimentally produced, characterized by deformed bones and extremely soluble skin. Cross-links increase in a tissue and some of them are converted to different types during the maturation process and aging.

Covalent interchain cross-links in collagen Types I, II and III result from the interactions of lysyl and hydroxylysyl derived aldehydes with other such aldehydes or with the ϵ amino group of these amino acids (Bornstein & Traub 1979; Light & Bailey 1980). The lysyl residues of the telopeptide regions (N-9 and C-17 of the α_1 chain and N-5 of the α_2 chain; see Fig. 1A) are converted to α-aminoadipic (δ)-semialdehyde, otherwise known as 'lysinal' (the aldehyde derivative of lysine; also referred to as allysine). The hydroxylysine is converted to 'hydroxylysinal' (hydroxyallysine; Bornstein and Traub, 1979). Lysyl oxidase which catalyzes this reaction appears to function preferentially on collagen aggregates or fibrils (Siegel, 1974) and prefers hydroxylysine as its substrate.

The intramolecular cross-links found in soft tissue collagens are in the N-

terminal telopeptide, between adjacent aldehydes N-9 to N-9 of two α_1(I) chains, or an α_1(I) and an α_2(I) (N-5) chains. The function of these cross-links is not known. Aldimine type cross-links involve lysinal in the C-terminal telopeptide (C-17) of one collagen molecule and a hydroxylysine within the helical portion of another collagen molecule (residue 87, in the α_1CB5 peptide; Fig. 1B), thus forming an intermolecular cross-link (dehydro-hydroxylysinonorleucine). The location of the hydroxyl group may be reversed in the above two participants in this cross-link, however a similar end product is obtained (Bailey and Peach, 1968). The adduct involving hydroxylysinal and lysine or hydroxylysine undergoes a spontaneous Amadori rearrangement to form a keto-amine, which is chemically more stable, the physiological significance of which is not known. Reduction of both dehydro-hydroxy-lysinonorleucine and the keto-amine with ^{3}H-sodium borohydride yields ^{3}H-hydroxylysinonorleucine, with the only difference being in the location of the tritium (Bornstein and Traub, 1979).

The reducible cross-links appear to decrease with ageing or maturity of the tissue/organism (Robins et al., 1973), while the number of non-reducible cross-links increases. Robins (1983) confirmed the existence of pyridinoline in sheep long bone collagen, and demonstrated that it probably exists *in vivo* as a galactosylpyridinoline. This type of cross-link was also demonstrated in Type II collagen from bovine articular cartilage (Robins and Duncan, 1983). The pyridinoline is involved in cross-linking three peptides from different chains, a reducible cross-link (between two α chains) and a hydroxylysine from a third

chain. This cross-link is thought to stabilize the 4D stagger of adjacent collagen molecules.

Intermolecular cross-linking involving the α_1CB6 peptide has been shown to involve two different specific interactions (Bornstein and Traub 1979). The residue C-17 is cross-linked to residue 87 of α_1CB5 (Fig. 1A). The other cross-link involves residue N-9 and residue 930 of the α_1CB6 respectively (Fig. 1B; Bornstein and Traub, 1979). Scott and Veis (1976b) analyzed acid insoluble bovine skin and dentine collagen after digesting with cyanogen bromide. They calculated that, on average, 1.5 cross-links per collagen molecule involved the α_1CB6 peptide. In insoluble bovine dentine collagen, Scott and Edwards (1981) showed that on average, there is one cross-link per molecule originating from the C-terminal telopeptide and one from the N-terminal telopeptide. Evidence for more complex cross-links involving lysyl, hydroxylysyl and histidine is also available (Yamauchi et al, 1987).

B.5. Supramolecular Assembly and Function of Collagen

According to the general theory (Schmitt et al., 1955), collagen molecules interact and align themselves linearly. As refined by Hodge and Petruska (1963), a collagen molecule in a stained and dried fibril is 4.4 D long, giving rise to an 0.4 D overlap and an 0.6 D gap region. This staggered array of molecules in the native fibril in tissues result in a characteristic striation with a periodicity of about 68 nm when seen under the electron microscope. However, there is no

unambiguous three-dimensional model yet available for the lateral packing of the molecules within the microfibril (Lee, 1987). Microfibrils assemble to form fibrils, which may range in diameter from about 50°A to several thousand Angstroms. The fibrils are stabilized by the cross-links described above. Fibrils in turn may assemble into fibers having a diameter of 0.1-14 μm.

Collagen serves primarily as a structural macromolecule in many different tissues of the organism. All tissues whose primary functions are either weight bearing (bone, cartilage), transmission of forces (tendon, ligaments), protection or compartmentalization (dermis, fascia), transmission of light (cornea, vitreous), or distribution of fluids (blood vessels, glandular ducts) contain collagen as the major macromolecular component.

Organization of collagen differs not only in various tissues but in some cases changes in the same tissue at different stages in the development and maturation of the organism. The architecture of the tissue appears to be determined in part by the predominant genetic collagen type and by the association of the collagen or collagens with other structural macromolecules, particularly proteoglycans and (acidic) glycoproteins. In tendon, composed almost entirely of type I collagen, fiber bundles are large and arranged in parallel, giving tensile strength as great as 20-30 Kg/mm^2 of tissue. Fibers in bone matrix permit the specific and regular association of hydroxyapatite crystals. In the skin collagen fibers are in a generally irregular and loose weave which allows for extensibility in all directions within the plane of the tissue. In cartilage, collagen functions in the form of a fibrillar network

to supply tensile strength and limit swelling of the proteoglycan component of this tissue. Type III collagen regulates the elasticity and flexibility of connective tissue.

B.6. Other Collagen Types and Their Functions

Type IV Collagen: is the major component of the basement membrane. It forms sheet like structures (Yurchenco & Schittny 1990) and functions as a selective filtration barrier for macromolecules for example in the kidney and placenta and also separates extracellular matrix from epithelial or endothelial cell layers as in gut, skin, cornea, lung and blood vessels. It is suggested that basement membranes create barriers that allow embryonic cells to segregate and differentiate into specific tissues. In adults they serve as molecular filters in capillaries and glomeruli preventing the passage of proteins and they provide the scaffolding that maintains normal tissue form during regeneration and growth. Type IV collagen is known to be resistant to specific vertebrate collagenases but is readily degraded by the 72- & 92-kDa gelatinases (Collier et al., 1988 & Wilhelm et al., 1989). Other proteinases that act on type IV collagen are elastase from neutrophils and macrophages and chymotrypsin-like serine proteinase from mast cells (Kalebic et al.,1983).

Type V Collagen: is present in the vicinity of basement membranes and as a component of type I collagen fibers (Mayne and Burgeson; 1987). It may function as a connector between basement membrane and stroma of vascular smooth muscle. Type V collagen is degraded by gelatinase isolated from various

sources. Sage et al. (1981) have shown that thrombin, a serine proteinase, is capable of degrading type V collagen. They proposed that thrombin may play a role in the degradation of this pericellular collagen during certain forms of tissue injury.

Type VI Collagen: is observed in most extracellular matrices and may play a role as an interface between the main collagen fibril network and the cells (Mayne and Burgeson; 1987).

Type XI collagen is a minor apparently fibrillar, collagen component of articular cartilage matrix (Vaughan et al., 1988). Type XII and XIII collagen were discovered at the cDNA level (Pihlajaniemi and Tamminen; 1990). The list of collagens in vertebrates is not closed. The newer collagens are found at low concentrations or expressed at specific times of development.

C. Noncollagenous Components of the Extracellular Matrix

C.1. Proteoglycans

Proteoglycans (PGs) are macromolecules which consist of a protein backbone to which one or more glycosaminoglycan chains are covalently attached. The protein "core" may also carry N- and/or O-linked oligosaccharides. They occur predominantly in extracellular matrices and cell surfaces. The glycosaminoglycans include chondroitin sulphate, keratan sulphate, heparan sulphate, dermatan sulphate and hyaluronic acid (HA)(Kennedy, J.F.; 1979). Except for keratan sulphate they are unbranched polysaccharides and all except hyaluronic acid,

occur in covalent linkages to protein. They are anionic due to the presence of carboxylate and/or sulphate groups.

The types and concentration of the proteoglycans are quite variable between the different connective tissues (Chakrabarti and Park; 1980). These macromolecules are found in relatively low concentrations except in cartilages. Proteoglycans play a role in the hydration and osmotic swelling of the connective tissue. Other properties attributed to the proteoglycans are viscoelasticity, modulation of access of materials to and from the cells and inhibition of calcification (Pearson, C.H. 1982). Proteoglycans may also play a role in adherence of platelets to collagen and in inhibition of cell-cell interactions.

The cartilage proteoglycan, which is the most studied, consists of a protein core (apparent M_r 220 kDa) which is heavily substituted with chondroitin sulphate and keratan sulphate and also carries N- and O- linked oligosaccharides. The GAGs are concentrated in domains along the protein core. The keratan sulphate chains occupy a region close to the N-terminus and the chondroitin sulphate chains are found along a large portion of the polypeptide backbone stretching toward the C-terminus. A large number of proteoglycan monomers can bind noncovalently to a single chain of HA. The resulting aggregate with its very high negative-charge density, attracts a large amount of water (Christner et al., 1978).

The dermatan sulphate proteoglycans (DS-PGs) are widely distributed in the extracellular matrix of skin, sclera, tendon, cartilage and other connective tissues. Two forms of dermatan sulphate proteoglycans, called DS-PG I (or biglycan) and

DS-PG II (or decorin), are known to date. DS-PG I has two GAGs and DS-PG II has one GAG. The major structural features of these molecules is a series of nine leucine-rich repeats. The protein core of decorin from bovine dermis has an apparent M_r of 43 kDa on SDS-polyacrylamide gels, and physicochemical and electron microscopic studies suggest that it possesses a globular tertiary structure, containing three S-S bonds (Scott et al., 1986). A single L-iduronic acid-rich dermatan sulphate chain is attached to Ser-4 (Chopra et al.,1985), and three N-linked oligosaccharides are attached to the large C-terminal CNBr peptide (Scott & Dodd, 1990). DS-PGs noncovalently bind to fibronectin, and inhibit the adhesion of fibroblasts to fibronectin. They also enhance cell-cell communication, via an effect on the gap junctions of cells.

The DS-PGs are seen as transversely oriented filaments spaced at regular intervals, approximately equal to the periodicity of the fibrils, located almost exclusively at the d bands of the collagen fibrils. In developing rat tail tendon, there is an inverse relationship between collagen fibre diameter and the amount of DS-PG II present on the surface of collagen fibrils (Scott & Orford 1981). A variety of observations indicate that DS-PGs noncovalently bind to collagen fibrils at a specific locus on the surface of the fibril and inhibit fibrillogenesis *in vitro* (Vogel et al, 1983; & Scott et al.,1986).

Heparan sulphate proteoglycans (HSPGs) are present on cell surfaces and basement membranes (reviewed by Gallagher et al, 1986). HSPGs may be bound to membrane receptors that could mediate endocytosis of the proteoglycan. In

basement membranes, HSPGs are bound by tight, non-covalent interactions of the core protein with other major basement membrane components such as collagen type IV and the glycoprotein laminin (Laurie et al, 1986). The heparan sulphate chains generally display weak affinities for basement membrane protein and their function might be to maintain the correct spatial organization of the structural elements. In the glomerular basement membrane, the acidic polysaccharides constitute a polyanionic permeability barrier which restricts the diffusion of serum proteins.

C.2. Fibronectin and Laminin

Fibronectins are a a class of high-molecular-weight glycoproteins (M_r = 210,000-260,000) that exhibit a variety of functions. Fibronectins are found in most body fluids, loose connective tissues, basement membranes, and granulation tissues (reviewed by Akiyama & Yamada, 1987). Fibronectins can function in several ways. (a) As a bridge between cells and other extracellular structural molecules such as collagens and proteoglycans, (b) As an attachment factor for cells, and (c) As a structural molecule itself as part of the extracellular matrix. All fibronectins share many important features including size, gross structure and biological activity. They contain 5 - 12% carbohydrate. Fibronectins contain polypeptide regions particularly sensitive to proteinases. Partial digestion of fibronectin by leukocyte elastase and other enzymes releases fragments with various biological activities (Carsons, 1989) not possessed by the intact molecule.

These include inhibition of cell attachment, augmentation of the opsonin-independent phagocytosis by monocytes, chemotactic activity for monocytes and stimulation of fibroblast DNA synthesis (Vartio, T. 1983).

Laminin (M_r = 850,000) consists of three large polypeptide chains which by many inter- and intrachain disulphide bonds form a unique cross-shaped component. Laminin binds to type IV collagen, heparan sulphate proteoglycan and to itself to create an integrated structure within the basement membrane. Laminin binds to triple helical domains of type IV collagen. It may function in cell-specific responses, causing secretory cells to become polarized, neural cells to extend axonlike processes, various cells to migrate, and a variety of cells to differentiate. There are several isoforms of laminin e.g. Laminin S, Laminin K, etc. Thus laminins are important structural and regulatory molecules (Martin & Timpl; 1987).

D. Degradation of Extracellular Matrix Components

Several proteinases belonging to different classes may be involved in the degradation of extracellular proteins. Proteinases are classified according to the reactive group at the active site.

D.1. Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) have an essential zinc at the active site. Most of the enzymes that can degrade connective tissue proteins at neutral pH

belong to the metalloproteinase class. They have certain common properties. Matrix metalloproteinases are secreted from connective tissue cells such as fibroblasts in an inactive proenzyme (zymogen) form and they can be activated *in vitro* by treatment either with proteinases such as trypsin or plasmin, or with organomercurials such as 4-aminophenylmercuric acetate (APMA), with a loss in molecular weight of 10-12 kDa. They are active at neutral pH, require intrinsic zinc and extrinsic calcium ions for full activity. Calcium functions as an enzyme activator and as a stabilizer of the tertiary structure of the enzyme at physiological temperatures (Seltzer et al., 1976). MMPs are inhibited by chelating agents but not by inhibitors of serine, cysteine, or aspartic proteinases. They are also inhibited by the plasma inhibitor α_2-macroglobulin and a specific tissue inhibitor of metalloproteinases, TIMP (Cawston et al., 1981) produced by the same cells. The MMPs can be classified as :

Collagenase (MMP-1) is defined as a metalloproteinase acting at neutral pH, with the primary function of degrading all three alpha chains in triple-helical collagen (genetic types I, II and III) at a single locus, namely between residue 775 (glycine) and residue 776 (leucine or isoleucine) (Gross et al.; 1974 and Miller et al.; 1976), giving rise to a N-terminal $TC_{3/4}$ and a C-terminal $TC_{1/4}$ fragment. At physiological temperatures these products denature and become susceptible to attack by other non-specific proteinases. The melting temperature of the $TC_{3/4}$ fragment is 32°C, while that of the $TC_{1/4}$ fragment is 29°C (Sakai and Gross, 1967). This enzyme was first demonstrated in primary cultures of tadpole tail

tissues by Gross and Lapiere in 1962. Two separate gene products, one of mesenchymal cell origin and the other of neutrophil origin, have been identified. The presence of collagenase has been demonstrated in nearly every tissue or pathological processes associated with collagen degradation (Harris and Cartwright, 1977). Collagenases have been demonstrated to be secreted by fibroblasts (Werb and Burleigh, 1974; Stricklin et al., 1977), polymorphonuclear leukocytes (Robertson et al., 1972; Murphy et al., 1977), macrophages (Werb and Gordon, 1975), and from proliferating rabbit epithelium in culture (Donoff et al., 1971). Collagenase produced by rat uterine cells in culture was found by Welgus et al., (1985) to degrade denatured collagen types I through V.

Gelatinase (MMP-2)/(MMP-9) degrades types IV, V and VII collagens and denatured interstitial collagens and hence may act synergistically with collagenases. Two separate gene products have been identified to date. The 92-kDa gelatinase-B is predominantly produced by neutrophils, macrophages, keratinocytes and a variety of tumor cells (Werb, 1989; & Hibbs et al.; 1987). The 72-kDa gelatinase-A is secreted by fibroblasts, some transformed cells (Werb, 1989 and Salo et al.; 1985) and osteoblast-like bone cells (Rifas et al; 1989). It also degrades type XI collagen (Murphy et al; 1981) and may be involved in the destruction of cartilage matrix in diseases such as osteoarthritis.

Stromelysin-1 (MMP-3) has a M_r of 57,000 and degrades proteoglycans, type IV collagen, fibronectin, laminin and gelatin, and removes N-terminal propeptides of type I procollagen (Galloway et al., 1983; Chin et al.; 1985 & Okada

et al.; 1986). MMP-3 also digests type IX collagen which has an important role in maintaining the structural integrity of cartilage (Okada et al. 1989). It also acts as an activator of procollagenase and has a broad pH optimum (Murphy et al., 1987). Human recombinant MMP-3 was shown to cleave types II, IX, X and XI collagens (Wu. et al.; 1991). Wu et al. (1991) have shown that stromelysin-1 acts as a "telopeptidase" in that it cleaves intact types II and XI collagens between the N-terminal cross-linking hydroxylysine residues and the helix. MMP-3 inactivates α_1-antitrypsin by specific cleavages (Chin et al, 1985). Two gene products have been identified, stromelysin-1 and -2.

Telopeptidase (MMP-4) was initially detected as an activity directed towards the type I collagen C-telopeptide in porcine explant medium (Goldberg & Scott, 1986). It was shown to cleave between the triple helix and the lysyl residue at position 17, which is involved in intermolecular cross-linking (Nakano & Scott 1987a). Nakano & Scott (1987b) also showed that telopeptidase was important in involuting rat uterus.

Matrix metalloproteinase-5 (MMP-5) degrades native 3/4-collagen fragments ($TC_{3/4}$) resulting from the cleavage of types I, II and III collagens by MMP-1 (Overall and Sodek, 1987). MMP-5 was detected in human gingival crevicular fluid at sites of periodontal inflammation (Overall and Sodek, 1987). Rat osteoblastic cells and fibroblasts from rat molar periodontal ligament and gingiva (Otsuka et al., 1988; Overall and Sodek, 1987) produce this enzyme. It is also active on gelatin. It is now believed to be the same as MMP2.

Matrix metalloproteinase-6 (MMP-6) degrades cartilage proteoglycan. It also cleaves the B chain of insulin at two bonds: Ala-Leu (residues 14-15) and Tyr-Leu (residues 16-17) (Azzo and Woessner, 1986). MMP-6 was first detected in human articular cartilage extracts (Sapolsky et al., 1976; Woessner and Seltzer, 1984). Its cellular origin(s) remains unknown. However, MMP-6 has been found in human polymorphonuclear leukocytes (Azzo and Woessner, 1986). The apparent molecular weight of the latent form is 55,000 and of the active form, 35,000 (Azzo and Woessner, 1986).

MMP-6 acts optimally at pH 5.3 but displays 40% of its maximum activity at pH 7.2 and so has significant activity at physiological pH (Azzo and Woessner, 1986). Except for its acidic pH optimum, this enzyme exhibits the classical properties of the matrix metalloproteinases and thus can be classified in this group of enzymes. MMP-6 is elevated to about three times the normal level in human osteoarthritic cartilage. It is also probably produced by gingival fibroblasts (Nakano and Scott, 1988).

Matrix metalloproteinase-7 (MMP-7) digests various components of the extracellular matrix including gelatins of type I, III, IV and V collagens, fibronectin and cartilage proteoglycan (Woessner and Taplin, 1988). MMP-7 cleaves the B chain of insulin at the same points as MMP-6. MMP-7 was first described in the involuting rat uterus (Sellers and Woessner, 1980). The cell origin of this metalloproteinase is unknown. Pro-MMP-7 has an apparent molecular weight of 28 kDa and can be activated by organomercurials, resulting in a 19-kDa active

enzyme (Woessner and Taplin, 1988).

Putative MetalloProteinase (PUMP): is also known as Matrilysin (Crabbe et al.; 1992). The cDNA has been isolated by screening a human tumor cDNA library with a transin (rat stromelysin) probe (Muller et al., 1988). Expression of Pump cDNA in COS cells leads to secretion of a protein of M_r 28,000 with latent proteinase activity. Activation of the latent form with organomercurials leads to active Pump species of M_r of 21,000 and 19,000 (Quantin et al., 1989). Active Pump degrades gelatins of type I, III, IV and V collagens, and fibronectin (Quantin et al., 1989). Furthermore, as for stromelysin, transin, Pump can activate proMMP-1. Pump is unique in that it lacks the C-terminal hemopexin-like domain possessed by the other MMPs. Its activity is inhibited by the tissue inhibitor of metalloproteinase (TIMP-1) (Murphy et al., 1991a). Postpartum rat uteri contain elevated levels of Pump mRNA (Quantin et al., 1989). It appears that Pump may be identical to the previously described uterine metalloproteinase, MMP-7 (Woessner and Taplin, 1988).

Neutrophil Collagenase (MMP-8) shows activity similar to interstitial collagenase (MMP-1). The latent form has a M_r of 75 000 and is activated to 58,000. This enzyme can be activated by gold thioglucose and gold thiomalate which do not activate interstitial collagenase from gingival explant cultures (Sorsa et al.; 1988). The enzyme is stored and secreted as specific granules in the polymorphonuclear leukocytes.

Stromelysin-2/Transin (MMP-10) is a gene than MMP-3 induced in rat

fibroblasts either exposed to growth factor or transformed by oncogenic viruses (Matrisian et al., 1986). There is 75% overall homology between amino acid sequences of rabbit stromelysin, human stromelysin and rat transin (Whitham et al., 1986). Its molecular weight and substrate specificities are similar to those of stromelysin. Thus transin is a rat homologue of stromelysin.

D.2. Lysosomal Intracellular Proteolysis

Lysosomes are a special group of cytoplasmic particles 0.2-0.4 μm in diameter, containing various acid hydrolases which form an intracellular digestive system. Lysosomes digest exogenous materials taken in by phagocytosis, including endogenous constituents segregated from the cell's own cytoplasm, and secretory substances contributed by secretion granules. After digestion by the lysosomal acid hydrolases, these materials are cleared from the lysosomes by diffusion or transport across the lysosomal membrane. In certain cell types, or in some pathological situations, bulk discharge of the lysosomal contents by exocytosis may serve as a clearing mechanism. This putative mechanism, the release of lysosomal enzymes, is believed to be important in the destruction of extracellular structures under certain conditions. Over fifty lysosomal enzymes are known to date.

Cathepsin B is a thiol proteinase found in lysosomes (for review see Barrett and Mcdonald. 1980). The molecular weight of cathepsin B from various organs and tissues lies in the range of 24,000-28,000. There are multiple forms of

cathepsin B with pI values within the pH range 4.5-5.6. Many proteins including haemoglobin, azocasein, immunoglobulin G, cartilage proteoglycans, bacterial cell wall proteins and oxidized B chain of insulin are degraded by cathepsin B. Cathepsin B degrades insoluble and soluble collagen at acidic pH, cleaving cross-linked regions (reviewed by Burleigh; 1977). It also cleaves the C-terminal telopeptide of acid soluble type I collagen (Scott & Pearson, 1981).

Cathepsin D was first isolated from cow spleen by Anson (1940). This enzyme is the major cellular carboxyl proteinase and is homologous with other carboxyl proteinases such as pepsin. It is a glycoprotein molecule with a molecular weight of 42,000 comprising a single polypeptide chain. It has a strong preference for cleavage near the hydrophobic amino acids. Cathepsin D cleaves relatively large polypeptides and has scarcely any activity on small synthetic peptides. Scott and Pearson, (1978; 1981) have reported that cathepsin D cleaves the α_1-chain of native type-I collagen within the extracellular C-terminal sequence on the N-terminal side of the δ-hydroxy-α-aminoadipic acid δ-semialdehyde residue involved in the formation of intermolecular cross-linkages.

Dean and Barrett (1976) emphasized that both cathepsin B and D are likely to be much more active in the lysosomal system than they appear in test tube experiments. The pH in lysosomes can fall as low as 4.7, which would permit a high activity level for most of the lysosomal enzymes. The lysosomal acid proteinases now known to be important for intracellular proteolysis are cathepsin B, D, E, H, L and N, and there are inhibitors for each of these enzymes at various

levels of specificity.

The lysosomal localization of cathepsin D, which has been thoroughly established by cell fractionation methods, was also done by immunohistochemical staining (Dingle et al.; 1973). The discharge of lysosomal enzymes by frustrated phagocytic cells is believed to play a role in the pathology of rheumatoid arthritis and other degenerative diseases, and in physiological bone resorption (de Duve, 1978). Baggiolini et al. (1980) designated a similar mechanism of enzyme release as "phagocytic enzyme release" and suggested that the release is triggered by the interaction of the phagocyte with a phagocytosable particle. In this situation the stimulus for release is also the potential substrate for the enzymes that are released.

D.3. Other Collagenolytic Proteinases

Polymorphonuclear leukocytes, which are transient but predominate in acute inflammatory lesions, secrete elastase, collagenase, cathepsin G, and plasminogen activator: these proteinases are potent and produced in sufficient quantity to degrade most of the surrounding connective tissue components. Thus these cells are programmed to degrade and remove host tissue. Polymorphonuclear leukocyte collagenase is immunologically (Hasty et al.; 1987 & Birkedal-Hansen et al.; 1988) and catalytically (Bennick & Hunt; 1967) distinct from fibroblast collagenase. Cathepsin G, which degrades denatured collagen and collagen telopeptides (Starkey, 1977; Starkey et al, 1977), can activate latent collagenase,

gelatinase and stromelysin (Okada & Nakanishi; 1989).

Macrophages, which are ubiquitous and function in both physiological and pathological states, are also known to produce elastase, collagenase, and plasminogen activator in addition to the potent lysosomal cathepsins. Collagenase production by macrophages can be stimulated with concanavalin A or by specific antigens in vitro (Wahl et al., 1975), and the cells probably release increased amounts of collagenase in response to various stimuli in vivo. Collagenase release by activated macrophages is limited, and may not be important in extracellular collagenolysis.

D.4. Possible Degradative Sequences of Collagen *in Vivo*

Elastase and cathepsin G, primarily produced by neutrophils and macrophages initially attack peptide bonds adjacent to the cross-links, rendering the "loosened" molecular aggregates more accessible to the collagenase (Barrett and Starkey, 1973; Barrett, 1981). In the case of interstitial collagens, it is thought that after the initial cleavage is made across the triple helix by collagenase, the reaction products simultaneously undergo denaturation at body temperature and subsequently degradation by gelatinase. It is generally believed that the later reaction may proceed extremely rapidly and the former may be the rate-limiting step of the overall reaction.

On the other hand, the rapid destruction of connective tissue proteins is often featured by the presence of massive numbers of macrophages, macrophage like

cells, or fibroblasts which engulf large amounts of banded collagen fragments. Thus under these circumstances tissue collagen fibres are merely split into phagocytosable size fragments by specific collagenase with or without the aid of elastase and/or cathepsin G.

There are hence various pathways of tissue collagen degradation in terms of its extent of extracellular degradation under normal physiologic and pathologic conditions. In the event that collagen is extracellularly degraded by gelatinase to the extent of diffusible peptides, they may be pinocytosed by various phagocytic cells for further degradation. Those banded collagen fiber fragments found in phagolysosomes are undoubtedly further digested by potent lysosomal enzymes to oligopeptides and amino acids.

D.5. Regulation of Matrix Metalloproteinases

The controlled breakdown of the macromolecular components of connective tissue matrices is a fundamental feature of the development and growth of an organism. Metalloproteinases produced by the connective tissue cells are tightly regulated at several levels, including gene expression and the extracellular activation and inhibition of the secreted enzymes.

Expression of these enzymes by connective tissue cells has been shown to be modulated by a number of cytokines (e.g. interleukin-1), growth factors and hormones, some of which may be specific to cell type and others which are of more general activity. Many of the cytokines and growth factors that are effective

are known to be products of monocytes/macrophages, including interleukin 1 (McCroskery et al.; 1985), tumor necrosis factor -α (Dayer et al.; 1985), platelet-derived growth factor (Bauer et al.; 1985), interferon-α and transforming growth factor -ß (Edwards et al.; 1987). A number of these effectors have been shown to be synthesized by connective tissue cells themselves, indicating the importance of autocrine regulatory mechanisms in connective tissue cells. Factors such as soluble products of activated lymphocytes (Dayer et al., 1977) phagocytosis of poorly digestible particles (Werb & Reynolds, 1974) and ingestion of red-cell products (Mainardi et al.; 1977) have been shown to stimulate collagenase production by synovial cells. Interaction between different factors also appears to significantly modify the responses of connective tissue cells.

The activity of matrix metalloproteinases is controlled extracellularly by at least two mechanisms: (a) They are secreted in a latent, inactive form (zymogen), and then activated outside the cell (Stricklin et al. 1977, & Stricklin et al. 1978, Wilhelm et al. 1987, & Collier et al. 1988). (b) Tissue Inhibitors of MetalloProteinases (TIMP), are specific inhibitors of matrix metalloproteinases (Wilhelm et al. 1986, Welgus et al. 1979, & Welgus & Stricklin 1983). TIMP-1, TIMP-2 and LIMP belong to this group of inhibitors (see later). Sellers et al (1980) noticed very little increase in the levels of collagenase in the media of resorbing bones when compared with that of untreated controls, but a significant reduction in the amount of TIMP synthesized by the explants induced to resorb suggesting that regulation occurred by increasing inhibitor levels. The accelerated breakdown occurring in certain

disease processes is thought to be due in part to an imbalance in the regulation of the metalloproteinases.

D.5.1. Latency of Matrix Metalloproteinases

In order to understand the critical role that MMPs play in the catabolism of both the collagenous and noncollagenous components of the extracellular matrix, it is essential to understand how they are activated. The latent form can be activated by physical (chaotropic agents), chemical (mercurials), and enzymatic (trypsin, plasmin) treatments.

Rheumatoid synovium produces both latent collagenase and plasminogen activator which converts plasminogen to plasmin. Plasmin is more effective in activating latent collagenase than trypsin. Production of both collagenase and plasminogen activator is inhibited by the glucocorticoid dexamethasone (10^{-9}M). Plasminogen is present in normal serum at a concentration of 50-100 mg/deciliter, and in normal synovial fluid and membranes at 10-20 mg/deciliter. In an inflammatory synovitis the permeability of synovial blood vessels is greatly increased to plasma proteins. Once produced, collagenase can bind to fibrils and could be activated in large quantities in a very short time to initiate rapid destruction of articular tissues. A similar mechanism could explain the resorption of uterine collagen in the post-partum period (Woessner; 1962), or the rapid development of trophic ulcers of skin in paralyzed patients (Peacock; 1967)

In contrast to latent collagenase synthesized by the connective tissue cells,

reversible collagenase-inhibitor complex may be found under certain pathological conditions when neutrophil collagenase is involved, such as the synovial fluid of rheumatoid arthritis (Nagai; 1973). In fact, a series of reports by McCartney and Tschesche (1980, 1981 & 1983) have provided evidence that neutrophil collagenase is an enzyme-inhibitor complex. The neutrophil collagenase is distinguished from other collagenases by the fact that it is stored in the specific granules (Robertson et al., 1972; Murphy et al., 1982, Harris et al., 1984) and is structurally distinct from other tissue collagenases (Hasty et al. 1984; Crespo et al.1988). Possible activation mechanisms of this unique collagenase in vitro and in vivo have been discussed by Weiss and Peppin (1986).

D.5.2. Activation of Matrix Metalloproteinases

The mechanism of activation of procollagenase has been studied in great detail. It was proposed that Cys^{73} in the propeptide domain of latent collagenase is cocrdinated to the active site zinc atom in a fashion that blocks the active site. All modes of activation lead to dissociation or modification of Cys^{73}. It is replaced by water with a subsequent exposure of the active site. Accordingly, when Cys^{73} is "on" the zinc, the activity of the enzyme is "off". Thus the dissociation of Cys^{73} from the zinc atom is viewed as the "switch" that leads to activation (Springman, et. al. 1990; Nagase, H. 1990a Van Wart & Birkedal-Hansen, 1990).

The propeptide domain that contains the critical cysteine residue and the catalytic domain that contains the zinc-binding site are the only two domains

common to all of the MMPs. The amino acid sequences surrounding both the critical cysteine and a region of the protein chains containing two of the putative histidine zinc-binding ligands are highly conserved in all of the MMPs (Fig. 2.). Trypsin cleaves proMMP-1 between Arg-36 & Asn-37, generating a major intermediate of 46 kDa (Grant et al. 1987), whereas proMMP-2 is not activated by trypsin (see discussion).

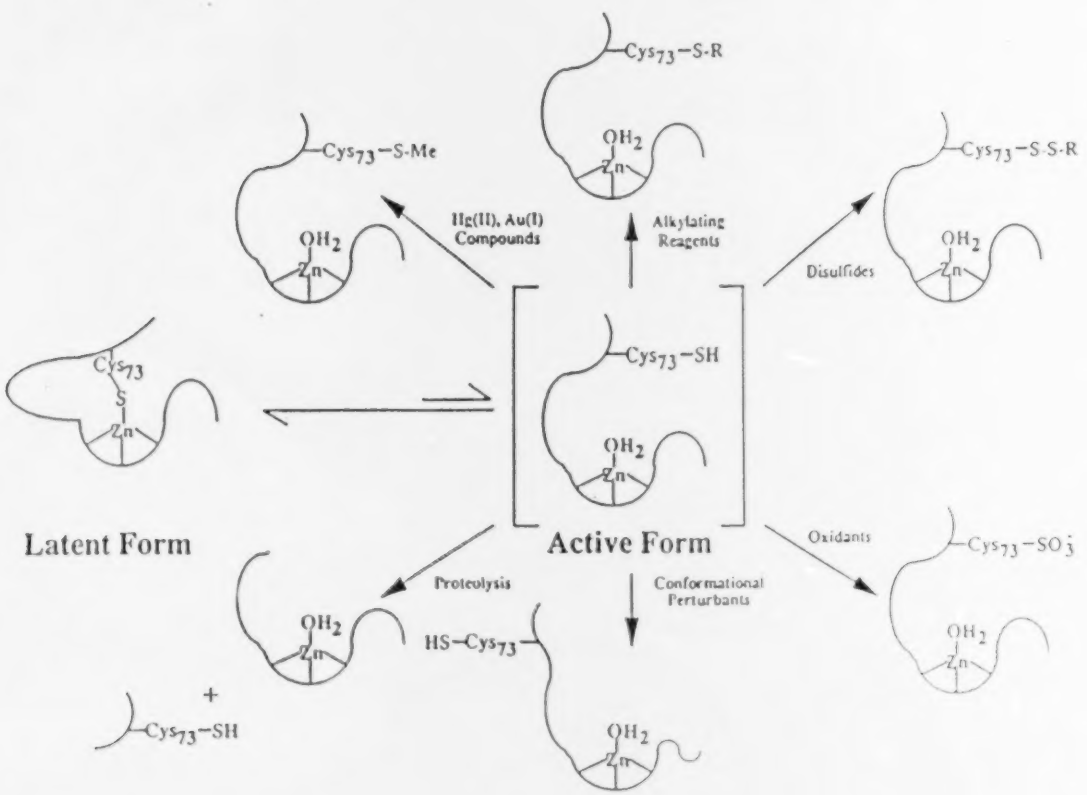


Fig 2. Model describing the multiple modes of activation of collagenase (Springman et al. 1990)

Treatment of pro-MMP-1 with organomercurials initially results in activation without loss of molecular mass (Stricklin et al. 1983, Grant et al. 1987). The intermediates are then converted into a 42-kDa active enzyme through an intramolecular autoproteolytic cleavage of one of the 3 bonds within the Gln^{80}-Phe^{81}-Val^{82}-Leu^{83} sequence at the amino terminus. Trypsin removes 84 amino acids from the amino terminal end of proMMP-3 (Wilhelm et al. 1987).

He et al. (1989) have described a proteolytic cascade leading to the complete activation of fibroblast procollagenase that may serve as a major physiological activation pathway. According to this view, epidermal keratinocytes secrete cytokines (interleukin 1, IL1) that stimulate fibroblast to produce procollagenase and prostromelysin. Keratinocytes also produce the urokinase type of plasminogen activator. Plasmin removes 81 residues from the amino terminal portion of procollagenase, leading to the formation of a partially activated species. Prostromelysin is also activated by plasmin, resulting in a 45-kDa active species. In the presence of active stromelysin, collagenase is further processed at the carboxyl-terminal end of the molecule, resulting in the fully activated enzyme.

While proteolysis may well be a physiological mode of activation of individual MMPs, nonproteolytic autoactivation may also have a role. Weiss & coworkers have shown that the latent 92 kDa type IV collagenase (Peppin & Weiss 1986) released by neutrophils can be activated by the HOCl that is produced from H_2O_2 and Cl^- by myeloperoxidase during the respiratory burst (Weiss et al. 1985).

Another physiological activation mechanism could involve oxidation by oxidized

glutathione through disulfide exchange, where its concentration is modulated by the glutathione cycle (Tschesche & Macartney 1981). The "cysteine-switch" model may allow flexibility in the way that an individual MMP is activated. Thus, one MMP may be more susceptible to activation by one mechanism than another. This may have important physiological implications in that it might allow for the selective activation of one or a small number of MMPs at certain sites. Alternatively the same MMP may be activated by different mechanisms in different cells or tissues.

D.5.3. Natural Inhibitors of Matrix Metalloproteinases

Naturally occurring inhibitors are thought to be involved in regulating the activity of MMPs *in vivo*. In addition to synthesizing proMMPs, various tissues and cells simultaneously produce an inhibitor of these enzymes. After latent collagenase (procollagenase) is separated from collagenase inhibitor in vitro, the enzyme undergoes autoactivation. It is possible that procollagenase is stable and not converted into active collagenase as long as it is accompanied by the inhibitor. Furthermore, the collagenase inhibitor may be a stabilizer of collagenase during its transport from cell to the extracellular site of action.

(a) Alpha$_2$Macroglobulin

Alpha$_2$macroglobulin (α_2M) is the principal circulating inhibitor of serine-, thiol-, carboxyl-, and metalloproteinases. It has four subunits and a molecular weight of 725,000 (Reviewed by Cawston T.E.; 1986). Alpha$_2$macroglobulin has the ability

to inhibit proteinases of all classes. The hydrolytic activity of the $\alpha_2$macroglobulin bound proteinases against low molecular weight substrates is retained. The α_2M-proteinase complex is rapidly cleared via receptors on macrophages and fibroblasts. The concentration of α_2M is relatively high in plasma.

The mechanism of inhibition ("Trap" mechanism) by alpha$_2$macroglobulin has been thoroughly studied. The proteinase causes limited proteolysis in the 'bait' region of α_2M, present near the middle of each subunit. This leads to a conformational change in α_2M resulting in the entrapment of the enzyme by α_2M and loss of enzyme activity towards large substrates. Alpha$_2$-macroglobulin accounts for 95% of collagenase inhibition. Collagenase binds to collagen like sequences on α_2M (Nagase; 1990. see discussion).

(b) Tissue Inhibitors of Metalloproteinases

TIMP 1 is a glycoprotein found in several tissues and tissue cultures. It has 184 amino acids, 13 hexose/mole, 6 disulphide bonds, a molecular weight of 28,500 and is weakly cationic (Cawston, T.E. 1986; Williamson et al., 1990). It is relatively stable to heat but is destroyed by high concentration of trypsin, and by reducing and alkylating agents (Murphy et al.; 1989). The complex with proteinase cannot be reversed by aminophenyl mercuric acetate or trypsin. TIMP 1 inhibits the proteolytic activity of all the metalloproteinases by forming a stoichiometric complex with the activated enzymes (Cawston et al., 1983).

TIMP 2 is less glycosylated then TIMP-1 with a molecular weight of 20,000.

MMP-9 is secreted as a complex of zymogen with TIMP-2 (Wilhelm et al.; 1989). It inhibits MMP-2 with a 1:1 stoichiometry. The MMP-active-site binding region of TIMP has been localized to the N-terminal domain (Murphy et al.; 1991).

LIMP Large inhibitor of matrix metalloproteinases has been described in rheumatoid synovial fluid (Cawston et al.; 1984) and bovine endothelial-cell culture medium (De Clerck & Laug.; 1986). Curry et al. (1992) have shown that the LIMP present in human lung fibroblast culture medium consists of 72000-M_r-progelatinase bound to TIMP-2. This inhibitor inhibits the three metalloproteinases collagenase, gelatinase and stromelysin, forming tight-binding complexes. This complex is not separated by high or low pH, even in the presence of EDTA, a metal chelator that disrupts the structure of progelatinase. Similarly 60°C temperatures in the presence of EDTA does not destroy the complex. It is suggested that the secretion of a complex (TIMP-progelatinase) in addition to a pro-MMP2 would restrict the activity of the active enzymes and also prevent uncontrolled connective tissue breakdown (Curry et al.; 1992). Both TIMP 1 (Schultz et al.; 1988) and TIMP 2 (De Clerck et al., 1991) have been shown to inhibit tumor cell invasion, probably due to their inhibitory effect on the activity of the two gelatinases.

E. Degradation of the Extracellular Matrix in Pathological States

E.1. Rheumatoid Arthritis

The mechanism of connective tissue destruction in pathological states has

perhaps been best studied in rheumatoid arthritis (Kelley et. al., 1981; Rothermich & Whisler, 1985). Rheumatoid arthritis is a chronic inflammatory disease of diarthroidal joints characterized by destructive and proliferative changes in synovial membrane, synovial fluid and articular cartilage. Eventually, joints are destroyed, ankylosed, and deformed.

An increase of collagen synthesis and deposition is seen in the joint capsule, and the net destruction of the cartilage are the major events in the progression of rheumatoid arthritis. Proteoglycan of the hyaline cartilage is depleted by various proteolytic enzymes such as elastase, cathepsin D, plasmin, and MMP-3 which accumulate in the synovial fluid. Collagenase activity is also detected in synovial fluids from some patients with rheumatoid arthritis (Harris et al.; 1970). Harris and McCroskery (1974) have shown that small increases in temperature significantly increase the susceptibility of type II collagen fibers to collagenase. They suggested that the difference demonstrated *in vitro* may have a physiological significance at the site of rheumatic fever.

E.2. Periodontal Disease

Periodontal disease is a chronic inflammatory condition of the supporting structure of the tooth: the periodontium, which is composed of cementum and alveolar bone connected by the collagenous periodontal membrane and the covering gingiva. Loe et al. (1965) have shown that periodontal diseases are caused by a local accumulation of multiple pathogenic bacteria. Like rheumatoid

arthritis, the cellular and molecular interactions of various soluble mediators generated by host tissue appear to destroy the host tissue itself, despite the fact that such responses evolved to protect the host from foreign antigens.

Although a few microorganisms at the disease lesions are known to produce bacterial collagenases, these are not believed to be of major importance in periodontal collagen breakdown (Genco and Slots, 1984). It has been demonstrated that inflamed as well as non-inflamed gingival tissues are capable of releasing substantial amounts of collagenase and other neutral proteinases (Uitto et al., 1981; Golub et al., 1974). Uitto et al.(1989) have shown that fibroblasts cultured with a partially purified 35 kDa trypsin-like protease from *Bacteroides gingivalis* secreted increased amounts of collagenase and plasminogen activator into the medium. They also showed that periodontal infection by *B. gingivalis* caused proteolytic damage of fibronectin a glycoprotein of host cell surfaces, extracellular matrix and plasma (Larjava et al, 1987). There was a marked increase in the concentration of immunoreactive granulocyte collagenase in crevicular fluids from patients with periodontal disease when compared to controls (Golub et al., 1976). Since the destruction of collagenous tissue is a main pathogenic feature of the disease, the possible role of collagenase in periodontal disease has been proposed (Birkedal-Hansen, 1980).

Heath et al.(1982) demonstrated that gingival tissue produced a group of metalloproteinases consisting of collagenase, gelatinase, and a proteoglycanase. From the same culture, they also isolated a specific metalloproteinase inhibitor

TIMP (see earlier). TIMP could be identified in the culture medium of gingival tissue removed from patients with no clinical evidence of periodontal disease but not in culture supernatants from diseased explants (Sellers et al., 1980). Meikle et al. (1986) have postulated the degradative process of periodontal tissue to occur as follows. The first step is the interaction of bacterial antigens with inflammatory cells, resulting in the production of a cytokine, interleukin-1. Non-inflammatory resident connective tissue fibroblasts are then induced by cytokines to produce metalloproteinases, such as collagenase and stromelysin and these enzymes cause the loss of connective tissue attachment and bone resorption in periodontal diseases.

E.3. Tumor Invasion

Proteinases play an important role in tumor invasion (Woolley, 1984; Liotta et al., 1984; Mignotti et al., 1986; Mainardi, 1987). The growth of neoplasms and metastasis of tumor cells involve the breakdown of surrounding barriers composed of connective tissue stroma and basement membranes.

Tumor explants induce normal tissue to produce collagenase *in vitro* (Matsumoto et al., 1979). In *in vivo* studies, the levels of collagenase appeared to be elevated (Wirl, 1977; Bauer et al. 1977) and the enzyme was concentrated at the advancing front of some tumors (Sakaki et al., 1976). Several serine and metalloproteinases, such as plasmin and stromelysin, are known to degrade type-IV collagen of the basement membrane. Soluble mediators such as interleukin-1

generated by primed host cells may function in these expressions. These factors may also be generated by tumor cells.

F. Synthetic Inhibitors of Matrix Metalloproteinases

Highly selective inhibitors of proteinases are potentially important therapeutic agents for control of the tissue destruction associated with various diseases. Thus inhibitors of pancreatic elastase (Powers & Tuhy, 1973), human leukocyte elastase and cathepsin G (Powers et al., 1977) were developed. Inhibitors were developed towards *Pseudomonas aeruginosa* elastase which is responsible for pulmonary emphysema and corneal infections (Nishino & Powers 1980). These inhibitors are substrate analogues, i.e. oligopeptides, with an attached chemical group which interacts with one of the active-site amino acid side-chains. The binding of the inhibitor is significantly strengthened and rendered specific, by the use of the oligopeptide sequence of a good substrate (i.e. one with high k_{cat}/K_m).

A number of very potent inhibitors have been isolated from culture filtrates of actinomycetes, including pepstatin, leupeptin, chymostatin, antipain, elastatinal and phosphoramidon (Umezawa & Aoyagi, 1977). Some of these appear to inhibit all enzymes sharing the same catalytic mechanism (e.g. pepstatin inhibits aspartic-proteinases), while others such as leupeptin, inhibit enzymes from more than one catalytic class, the determinant of selectivity being the requirement of the enzyme for certain amino acid residues or structurally-related groups that bind at the enzymes active site and subsites (see Fig. 3).

Several of the small natural inhibitors are aldehydes. These bind reversibly, but often very tightly, and are effective because the aldehyde group can form tetrahedral adducts with catalytic groups such as the serine hydroxyl or cysteine thiol. These structures mimic the postulated tetrahedral intermediates. Inhibitors of this type with activity against papain, chymotrypsin and elastase, have been synthesized (Kam et al., 1979).

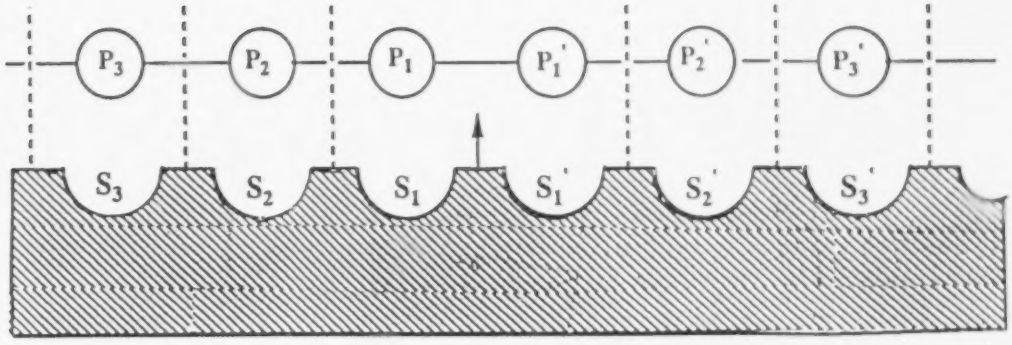


Fig 3. Diagram showing the subsites of proteinases and the complementary features of the substrate. The substrate-binding subsites are considered to be located on either side of the catalytic group, in the active site cleft. The subsites are numbered S_1, S_2, etc., away from the catalytic site towards the N-terminus of the substrate, and S_1', S_2', etc., towards the C-terminus. The corresponding residues (belonging to the substrate or inhibitor) that fit into the subsites are numbered as P_1, P_2 etc., and P_1', P_2', etc. The subsites are usually thought of as binding amino acid sidechains, but there are also important interactions with the polypeptide backbone, and with terminal blocking groups of synthetic substrates (adapted from Schecter and Berger, 1967).

The design of synthetic inhibitors for collagenase is hindered not only because the 3-dimensional structure of the enzyme is presently unknown but also because details of the interaction between collagen and collagenase have not been identified. No inhibition studies were reported on gelatinase at the start of this project probably because the role of gelatinase in tumor invasion was not known. Since telopeptidase, collagenase and gelatinase are metalloproteinases, natural and synthetic inhibitors of this class of enzymes are of particular interest. Phosphoramidon [N-(α-L-rhamnopyranosyloxyhydroxyphosphinyl)-L-Leucyl-L-tryptophan] is a potent inhibitor of thermolysin (the most extensively studied metalloproteinase). The oxygen of the phosphinyl group ligates the active site zinc thus inhibiting the enzyme (Umezawa & Aoyagi, 1977). Removal of the rhamnose by mild alkaline hydrolysis generates a phosphoramidate which is an even stronger inhibitor. Several phosphoryl-peptide (phosphoramidate) inhibitors have been synthesized (Kam et al., 1979). Other zinc-coordinating ligands, such as the hydroxamic acid, N-hydroxy- and thiol functional groups, are also effective when linked to short peptides (Nishino & Powers, 1979). One of these has been used for the affinity-purification of thermolysin (Nishino & Powers, 1979).

MMP-3 has been implicated in diseases such as rheumatoid arthritis (Okada et al., 1989b) and osteoarthritis (Martel-Pelletier et. al. 1984; & Dean et al., 1989). The early depletion of proteoglycans from cartilage coincides with the appearance of stromelysin in arthritic joints. Both stromelysin and collagenase are evident within 48 hrs of the onset of inflammation induced in animal models (Hasty et al.

1990). MPP-2 is presumably required for the turnover of basement membrane in vivo, but increased secretion has also been shown to be linked with the invasive potential of tumor cells (Liotta et al.1986., Tryggvason et al. 1987., Zucker et al., 1990). The latter may significantly facilitate the penetration of disseminating tumor cells through tissue compartments of the body. Thus inhibitors to MMP-2 would be of significant value.

Highly selective inhibitors to all three enzymes could be of great therapeutic use for control of the tissue destruction associated with various diseases, although the main objective of inhibitor synthesis in this study was to demonstrate that a selective inhibitor towards telopeptidase (a MMP which specifically cleaves the C-telopeptide of collagen) would inhibit the breakdown of fibrillar collagen by fibroblasts which are capable of secreting a range of MMPs.

The design of inhibitors to be synthesized in this study was based on the idea that product analogs with a chelating group (eg. hydroxamate) on the amino- or the carboxy- terminal would bind strongly in the active site of the enzyme and inhibit it. To make such inhibitors it is essential to know the scissile bond of the enzyme, or to know the structure of the active site of the enzyme (see Fig. 3). In the absence of any closely related structures, several research groups are now trying to obtain a crystal structure of a representative member of the MMP family (Lloyd et al.; 1989). Thermolysin *(Bacillus thermolyticus)*, a zinc metalloproteinase which has been studied extensively by X-ray crystallography is used by most investigators as a model for matrix metalloproteinases. Matthews (1988) studied

the crystal structure of thermolysin with a hydroxamic acid inhibitor and suggest that the hydroxamate group of the inhibitor binds in a bidentate fashion to the zinc at the active site of the enzyme (Fig 4). Zincov [2-(N-hydroxy-carboxamido)-4-methylpentanoyl-L-alanyl-glycine amide] a commercially available inhibitor of thermolysin was found to inhibit partially purified telopeptidase from gingival explant culture medium (25% inhibition at a concentration of 0.3 mM; Nikrad, M.P. and Scott, P.G. unpublished results).

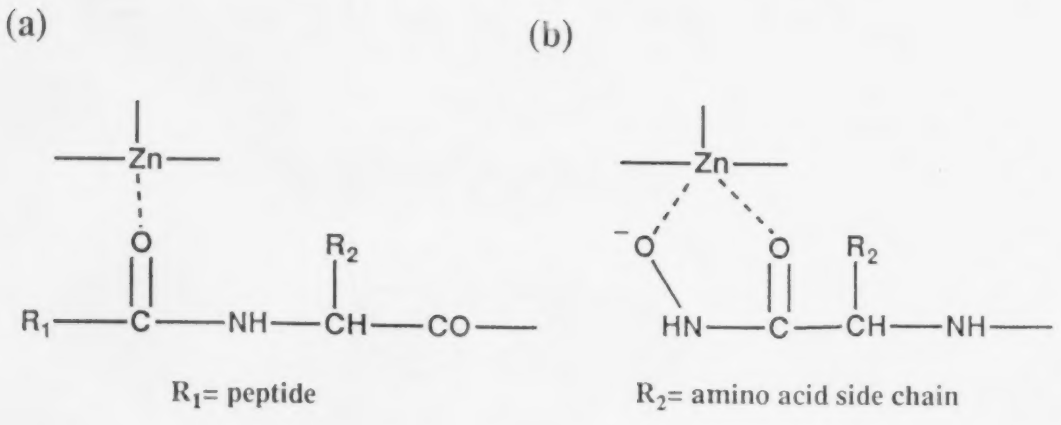


Fig 4. Schematic diagram showing the active site zinc interacting with (a) substrate and (b) hydroxamate inhibitor (pk_a of hydroxamate group is 6.0).

G. Object

As described in the Introduction it is uncertain as to which enzyme is responsible for the onset of fibrillar collagen breakdown. Collagenase was shown to have a minimal effect on insoluble cross-linked collagen (Leibovich and Weiss; 1971). Vater and Harris (1979) showed that by increasing cross-links in collagen using lysyl oxidase its breakdown by collagenase *in vitro* was reduced greatly. Hence it was suggested that there may be a distinct enzyme(s) capable of releasing collagen monomers or aggregates from fibrillar collagen. Rauterberg (1973) suggested that intermolecular cross-links originating from the amino- and carboxy- terminal telopeptides may modulate the degradation of fibrillar collagen. Harris and Cartwright (1977) proposed that the removal of these cross-links may be a prerequisite for effective collagen turnover. A variety of lysosomal enzymes, including cathepsins B, D, N and L have activity against the telopeptides effectively releasing the cross-links. Because these enzymes all have acidic pH optima it is unlikely that they would play a role in extracellular collagen breakdown under normal physiological conditions (pH 7-8). They may instead play a role in the intracellular digestion of phagocytosed fibril fragments. Polymorphonuclear leukocyte elastase, a neutral serine proteinase is quite effective in solubilizing fibrillar collagen. This enzyme may be very important in inflamed tissue, but collagen is also degraded during normal processes such as uterine involution, tadpole tail resorption, cervical dilation, angiogenesis and wound healing. Scott et al. (1983) have shown that gingival fibroblasts produce an enzyme

("Telopeptidase"/MMP-4) which cleaves within the C-terminal nonhelical region of collagen.

If the above is indeed the major role of telopeptidase, it should be possible to make specific inhibitors to this enzyme and to show, in tissue culture, the inhibition of the breakdown of fibrillar collagen in the presence of other matrix metalloproteinases. Specific inhibitors to other matrix metalloproteinases would also be useful. For example, gelatinase (type IV collagenase/MMP-2) may have an important role in tumour metastasis and inhibitors of this enzyme might help inhibit tumour invasion.

The objective of this project was to make such inhibitors. To do so it is important to have information regarding the enzyme of interest, namely:

(1) The class to which the enzyme belongs (knowing this one could perhaps make use of the X-ray structure and inhibitor study done for other enzymes from the same class to design inhibitors); and

(2) Its substrate specificity. With this information one can make inhibitors that are analogues of the product or substrate to improve binding.

Telopeptidase is a metalloproteinase since its activity is inhibited by chelating agents such as 1,10-phenanthroline, but not by serine and thiol proteinase inhibitors such as phenylmethyl sulphonyl fluoride and N-ethylmaleimide (Nakano & Scott 1987a). Attempts would be made to identify the scissile bond(s) of telopeptidase in the C-telopeptide of collagen, which would require the purification of the enzyme from culture medium and the synthesis of peptide substrate(s)

corresponding to the C-telopeptide of collagen. Peptide hydroxamates were made by Nishino and Powers (1978) as inhibitors of thermolysin, possibly the most extensively studied metalloproteinase. Zincov [2-(N-hydroxy-carboxamido)-4-methylpentanoyl-L-alanyl-glycine amide], a commercially available inhibitor of thermolysin, inhibits telopeptidase (MMP-4) and collagenase (MMP-1) to some extent (see Introduction). Based on the available information it was decided to make di-, tri- and tetrapeptide hydroxamates. These would then be tested against telopeptidase, collagenase, gelatinase (MMP-2) and stromelysin (MMP-3). The length and the sequence of the peptides could then be changed for individual metalloproteinases according to their specificity.

II. MATERIALS AND METHODS

A. MATERIALS

A.1. Column Chromatography

Bovine serum albumin, Coomassie Brilliant Blue G (for protein assay), TPCK-[L-(tosylamido 2 phenyl) ethyl chloromethyl ketone]-treated trypsin, azocasein, trypsin from bovine pancreas (50 units) bound to agarose were obtained from Sigma Chemical Co., (St.Louis, MO.). Trypsin (270 units/mg) was from Worthington. Azocoll (50-100 mesh) was obtained from Calbiochem-Behring, (San Diego, Ca.). PBE^{TM} 94 and Polybuffer^{TM} 74 (for chromatofocusing), PhenylSepharose CL-4B (for hydrophobic interaction chromatography), PD-10 (Sephadex^{TM} G-25M) desalting columns were from Pharmacia (Canada) Ltd., Dorval, Que. Imidazole was from BDH Ltd., Toronto, Ont. Ethylene glycol was from Caledon Labs Ltd., Georgetown, Ont. Ammonium sulphate, analytical grade was from Serva / Feinbiochemica. Bio-Gel A-0.5m, urea, Tris, and dithiothreitol were from Bio-Rad Labs Ltd., Mississauga, Ont. Nuclear-Chicago tissue solubilizer and Liquifluor liquid scintillation fluid were from Dupont Canada Inc., Markham, Ont. Sodium azide, Brij 35, and other reagent grade chemicals were obtained from Fisher Scientific, Edmonton, Alta. Proteodermatan sulphate was isolated from mature bovine skin, purified, and characterized as described by Pearson et al., (1983).

A.2. Polyacrylamide Gel Electrophoresis

Polyacrylamide, BIS(N,N' methylene-bis-acrylamide), SDS (sodium dodecyl sulphate), TEMED(N,N,N',N-tetramethyethylene diamine), ammonium persulphate, Bromophenol Blue, Coomassie Blue (R 250), Tris(tris (hydroxymethyl) amino methane) and the mixed bed ion exchange resin AG 501-X8(D) (20-50 mesh) were obtained from Bio-Rad Laboratories (Mississauga, Ont.)

A.3. High Performance Liquid Chromatography

The Gilson HPLC, used for peptide separations, consisting of model 302 pumps and an Apple II plus microcomputer controller and gradient manager 702v 1.2 software, was obtained from Gilson Medical Electronics, Middletown, WI, U.S.A. A variable wavelength UV detector (model ERC 7210), used with the Gilson chromatography system, was from Erma Optical Works (Tokyo, Japan). FRAC-100 fraction collector was from Pharmacia. HPLC grade acetone, acetonitrile and methanol were from Caledon Labs, Georgetown, Ont. Trifloroacetic acid and heptafluorobutyric acid were from Pierce Chemical Company (Rockford, Illinois USA). Vydac RP-201-TP, and RP-218-TP reversed-phase columns were from The Separations group, Hesperia, CA, USA. The cation exchange column SynChropak S-300 (containing sulphonyl reactive groups) and the reversed phase column Synchropak RP were from SynChrom, Inc. (Lafayette). Peptide standards for reversed phase chromatography and cation exchange chromatography were purchased from Alberta Peptide Institute, Edmonton.

A.4. Peptide Synthesis

Benzhydrylamine Resin (0.85 mmoles/gm) was purchased from Bachem (3132 Kashiwa street, Torrance, Calif. 90505). Most of the amino acids were from Institut Armand-Frappier (Montreal, Que.). The protected amino acids groups were Boc-Tyr(Br-Z), Boc-His(Tos), t.Boc-Ala, t.Boc-Lys(2-Chlorocarbobenzoxy), t.Boc-Glu(OBzl), Boc-Gln, Boc-Proline, Boc-Arg(Tos), Boc-Asp(OBzl). The coupling agent N,N'-dicyclohexylcarbodiimide, N-Hydroxybenzotriazole and N-Hydroxysuccinimide were from Sigma Chemical Co., (St. Louis, MO.).

Z-Leu-Pro-Leu-Gly, Z-Gly-Pro-Leu-Gly, Z-Pro-Gln-Gly and Ac-Pro-Leu-Gly were synthesized on a Beckman model 990 peptide synthesizer (in Dr.R. S. Hodges laboratory in the Department of Biochemistry.) using the Merrifield peptide resin [1% x-linked; 0.9mmoles/gm, No. 24610, Pierce], and the t-Boc solid phase peptide synthesis approach (Stewart and Young; 1984).

A.5. Amino Acid Analysis

Amino acid analyses were done by the Waters PICO TAG™ method after hydrolysis with 6N HCl in evacuated and sealed tubes, for 22-24 hours at 110°C. The derivatives were separated using Beckman 110A pumps and Waters PICO TAG columns (3.9mm x 15cm).

A.6. Radioisotopes

^{14}C-acetic anhydride(119mCi/mMole) and ^{3}H-sodium borohydride

(110mCi/mmole) were obtained from Amersham/Searle Co. (Oakville, Ont.). Tritiated water (0.25mCi/g), ^{3}H-sodium borohydride (25mCi/mmole), and scintillation fluid was obtained from Dupont Canada Inc., (Mississauga, Ont.).

A.7. Other Reagents and Products

Ammonium sulphate (enzyme grade) and ß-D(+)Glucose were from Sigma Chemical Co. (St. Louis, MO.). Formalin was from Fisher Scientific, Edmonton, Alberta. Cyanogen bromide was purchased from Aldrich Chemical Co, and stored at -20°C. Gelatin was obtained from J.T. Baker Chemical Co. (Phillipsburg, New Jersey). Other chemicals and disposables were obtained from Fisher Scientific, and CanLab, Edmonton, Alberta. The water was either double distilled, or singly distilled and further purified through a Milli-Q™ system with a 0.22μm Milli-Stack™ filter (Millipore, Ltd. Mississaugua, Ont.).

B. METHODS

B.1. Isolation and Purification of Collagen Substrates

B.1.1. Preparation of Soluble Bovine Skin Collagen

Acid soluble collagen (Type I) was prepared from the corium layer of a freshly flayed foetal bovine skin (Canada Packers, Edmonton) according to the protocol of Volpin and Veis (1971). All the extractant solutions contained the following in a final concentration: 0.003M-sodium azide, 0.005M-NEM, 0.005M-benzamidine hydrochloride, 0.001M 6-amino hexanoic acid and 0.01M-EDTA. During the entire

procedure, the skin or extractant solutions that contained the collagen were kept at 4°C.

The foetal bovine skin was washed in 10% (w/v) NaCl containing the inhibitors and left for 2 days in the above solution with one change. The hair and outer layer of skin, as well as any subdermal adipose tissue were scraped off, and then the skin was chopped into pieces of 2-3mm^3. These sections were suspended in the 10%-NaCl solution for several days with changes, and then suspended in 25% NaCl for 24 hours. The salt was removed by extraction in water over several days.

Acid soluble collagen (Type I; Type III collagen is not extractable in dilute acids) was obtained from these 24 hour exractions with 0.5N-acetic acid. The extracts from each day were clarified by centrifugation (8,000 x g; 1 hour). The collagen in these extracts was precipitated by the addition of solid NaCl to a final concentration of 10% (w/v). After 24 hours, the precipitate was collected by centrifugation (17,000 x g; 1hour). Additional NaCl (5%; w/v) was added to the supernatants to ensure maximum recovery of the collagen. The precipitated collagen was redissolved in 0.5N-acetic acid overnight, the solution clarified by centrifugation (17,000 x g), and collagen was reprecipitated by addition of an equal volume of 30% (w/v) NaCl. The precipitated collagen was recovered by centrifugation. The two steps were performed a total of 3 times, ending with the collagen in solution. The extracts from each day were handled separately to quantitate recoveries. The final collagen solutions were dialyzed exhaustively against water without the proteinase inhibitors, lyophilized and stored at 4°C. This

was called 'acetic acid soluble collagen'.

The acetic acid soluble collagen was purified further to remove any bound proteinases (Pardo et al., 1980) by the method described by Gross (1958). The lyophilized collagen (10-40mg) was suspended in 0.5 M acetic acid at a concentration of 2mg/mL, and dissolved overnight at 4°C. The collagen solution was dialyzed against 2 changes of 0.1 M sodium phosphate buffer pH 7.6, and the concentration adjusted to approximately 1.0mg/mL with fresh phosphate buffer. Trichloroacetic acid (TCA; 25% solution in 0.45 M NaCl) was added to a final concentration of 2.5% and to pH of approximately 3.5 (never lower than pH 3.0). After 45 minutes the resultant precipitate was centrifuged at 20,000 x g for 45 minutes at 4°C. The supernatant was dialyzed against 0.1M-sodium phosphate pH 7.6, for 48 hours with 2 changes. Ethanol (95%) was added to give a final concentration of 14%(v/v). The collagen was allowed to precipitate out of solution overnight and then centrifuged at 30,000 x g. The collagen was resuspended in 0.5N-acetic acid, dissolved overnight, and then dialyzed against 0.01N-acetic acid. An aliquot was used for the measurement of the collagen concentration by the microbiuret assay as described below. Recoveries of the purified collagen were generally between 50-75%.

B.1.2. Labelling of Acetic Acid Soluble Collagen with ^{3}H-Sodium Borohydride

The acetic acid soluble fetal bovine skin collagen used had been extracted with the proteinase inhibitors and purified with TCA and ethanol precipitation as

described above. This collagen was kept on ice throughout the following procedure. The collagen, at a concentration of 1mg/mL in 0.01N acetic acid, was neutralized by addition of 0.5M sodium phosphate, pH 7.0, to a final concentration of 0.05M-sodium phosphate. Tritiated sodium borohydride was diluted with cold sodium borohydride in a 1:1 ratio, and approximately 1mg of this was added to the collagen with mixing for 1 or 2 minutes. Five milligrams of collagen was reduced at a time. Glacial acetic acid was then added to lower the pH to 5 and destroy the reductant. The solution was left for 30 minutes to allow for the tritiated gas to dissipitate, and then it was dialyzed on alternate days against large volumes (2 litres) of 0.02 M sodium borate, pH 9.0 (to decrease the non-specific label on the collagen), and 0.01 M acetic acid for several days (until the radioactivity in the dialysate was close to background) with daily changes. The collagen (0.5mg/mL to 1.0mg/mL) was dispensed in 1.0 mL aliquots in 7.0mL scintillation vials and stored at -20°C (Fig 5).

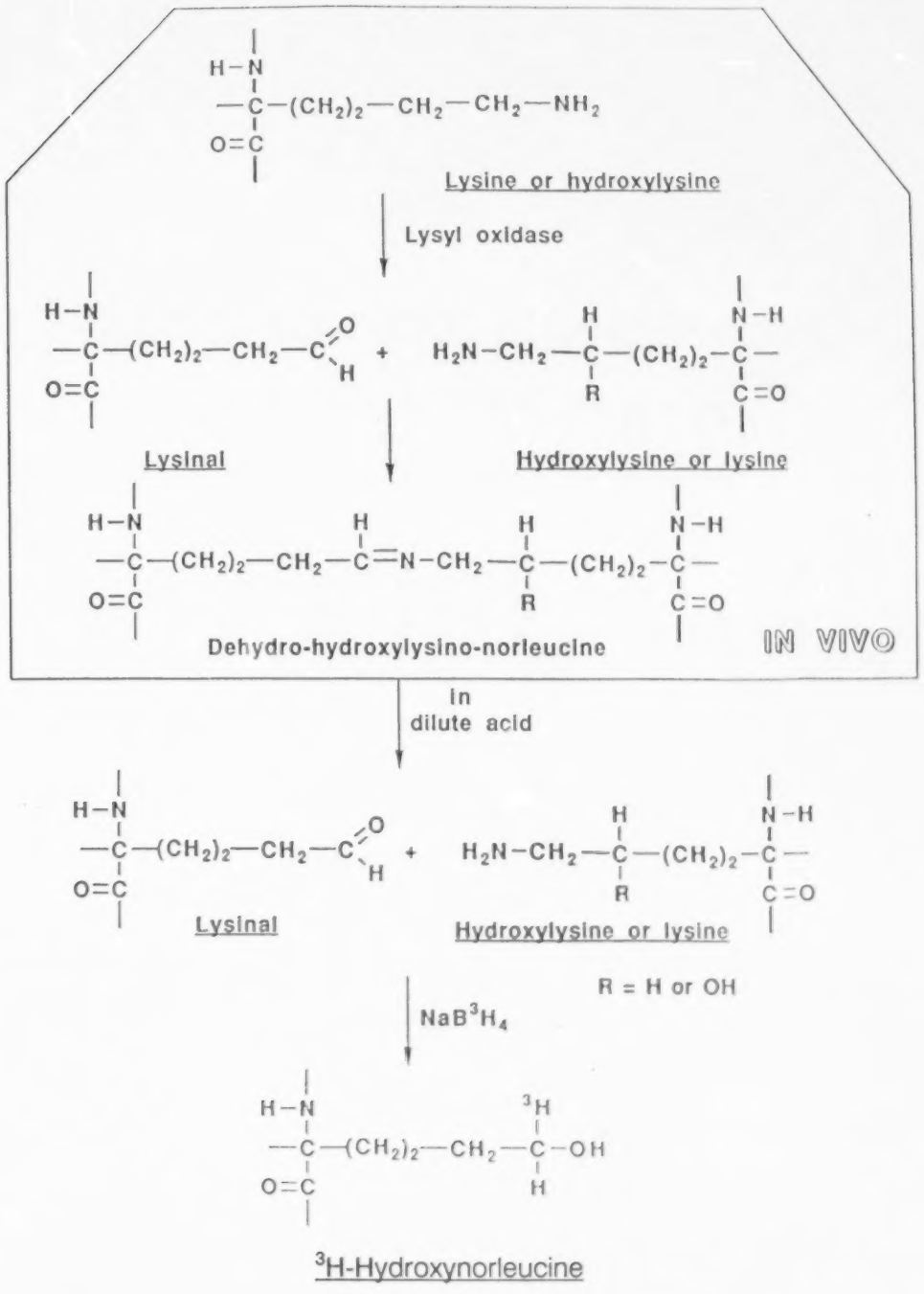


Fig 5. Labelling of Acid Soluble Collagen with ^{3}H-Sodium Borohydride

B.1.3. ^{14}C-Acetylation of Acetic Acid Soluble Collagen

An aliquot (10mg) of the acid soluble collagen extracted with proteinase inhibitors as described above, was dissolved in 0.1N acetic acid at 4°C. The solution was dialyzed against 0.1M sodium phosphate pH 8.8 for 2 days. The collagen (1.6mg/mL) was transferred to a thick walled container, and kept in a cold water bath to maintain the temperature between 8 and 12°C throughout the following procedure. The contents of an ampule containing 500μCi of ^{14}C-acetic anhydride (119mCi/mMole), were diluted with 150μL of a 1% solution of cold carrier acetic anhydride in benzene, and transferred by washing with 350μL benzene. An aliquot of 60μL of this solution was added to the collagen at zero time and at every 30 minutes for a total of 5 additions. The solution was kept on ice for a further 1.5 hours. The acetylated collagen was then dialyzed against 0.01N-acetic acid for several days until no further radioactivity was released. The ^{14}C-collagen was stored at -20°C.

B.1.4. Culture of Gingival Fibroblasts

Samples of clinically normal gingival tissue removed during periodontal surgery and biopsies of human skin were placed into explant culture. Fibroblasts growing out were harvested and passaged. The materials and methods for tissue culture are described in detail elsewhere (Nakano & Scott 1986). The growth medium (DMEM-10) was Dulbecco's modified Eagle medium, supplemented with 10% (v/v) fetal calf serum, HEPES (4.7 g/L), $NaHCO_3$ (3.7 g/L), penicillin-G (100 mg/L),

gentamycin sulfate (50 mg/L), and fungizone (20 mg/L).

B.1.5. Labelling of Collagen Fibrils with Fluorescein Isothiocyanate

Insoluble bovine Achilles tendon type I collagen (Sigma) was labelled with fluorescein isothiocyanate (FITC) (Sigma) (Steven and Lowther, 1975). After washing 4 times each with water, methanol (50% v/v) and acetone to remove as much unbound fluor as possible, the collagen was resuspended at about 1 mg/mL in 50% methanol containing 1% (v/v) glacial acetic acid by repeating homogenization for a few seconds using a Polytron™ homogenizer. Any large pieces of collagen were removed by centrifugation at 1000 rpm for 2 min. The suspension could be stored for at least 3 months at 4°C in foil-wrapped containers before use. An aliquot (250 μL) of this suspension was added to each well of a 6-well tissue culture plate which was then allowed to dry uncovered in a sterile atmosphere for 1 hour. Coated plates were stored in the dark for up to 1 month at room temperature before use.

B.2. Polyacrylamide Gel Electrophoresis

B.2.1. SDS Polyacrylamide Gel Electrophoresis (5%)

Polyacrylamide gel electrophoresis was performed as described by Furthmayr and Timpl (1971), as modified by Scott and Veis (1976a). Stock solutions of the following were prepared, filtered and stored. A concentrated stock buffer consisted of a 0.4M-sodium phosphate, 0.3M-sodium hydroxide, 0.08% (w/v)

sodium dodecyl sulphate (SDS) adjusted to pH 7.2 with 2N-NaOH, and stored at room temperature. The electrophoresis running buffer was a 1:8 dilution of the stock buffer. The stock acrylamide solution consisted of 29.1% (w/v) acrylamide and 0.9% (w/v) BIS stored in the dark at 4°C. The sample buffer was 0.01M-sodium phosphate containing 0.22% (w/v) SDS and 2.0M-urea and stored at 4°C. The 0.1(w/v) Bromophenol Blue in water, was stored at 4°C.

For the preparation of 5% polyacrylamide gels, 5.7mL of the acrylamide monomer solution was mixed with 4.25mL of the stock buffer and the volume made up to 32.25mL with water. Ammonium persulphate (1.7mL of a 5mg/mL solution freshly prepared), and TEMED (51μL) were added with mixing. The solution was immediately dispensed into glass tubes (0.55 x 12.5cm) and filled to 10.5cm. N-butanol was applied to the top of each gel, and the gels left for 45 minutes to polymerize.

To the samples 20-50μL (if liquid) or 20-30μg (if solid), 40μL of sample buffer was added and heated to 50°C for 30 minutes or at 80°C for 5 minutes. Bromophenol Blue (10-15μL) was added to each sample, and this solution was applied on top of each polyacrylamide gel under the running buffer.

Electrophoresis was carried out for 45 minutes at 1mA per gel, or until the sample had entered the gels, then at 3mA/gel for 15 minutes and finally at 6mA/gel for approximately 3.5 hours or until the tracking dye was within 1cm of the end of the gel. The gels were then carefully reamed out with water using a syringe fitted with a 4cm 26 gauge needle and India ink was injected into the

center of the tracker dye band. The gels were stained overnight in a solution of 25% (v/v) isopropanol, 10% (v/v) acetic acid, with 450mg Coomassie Blue, made up to 1 litre with water. The gels were destained for 12-36 hours in a diffusion destainer containing the ion-exchange resin AG501-X8(D), 20-50 mesh, and 10% (v/v) acetic acid.

B.2.2. SDS Polyacrylamide Gel Electrophoresis (12%)

This system employs the use of a stacking gel (3%) on top of a 12% separating gel, according to the method of Laemmli (1970). Stock sclutions of the following were prepared, filtered and stored. The separation gel buffer (4 x concentrated) consisted of 1.5 M Tris, 0.4% (w/v) SDS and was adjusted to pH 8.8 with HCl. The stacking gel buffer (4 x concentrated) contained 0.5 M Tris, 0.4% (w/v) SDS and was adjusted to pH 6.8 with HCl. The running buffer (8 x concentrated) consisted of 0.2 M Tris, 1.53 M glycine, 0.8% (w/v) SDS and adjusted to pH 8.3 with HCl. The sample buffer was 0.0625 M Tris/HCl, 2% (w/v) SDS, 2.0 M Urea, pH adjusted to 6.8. The catalyst, stock acrylamide, tracker dye and staining and destaining solutions were as for the 5% SDS polyacrylamide gels.

Stock acrylamide (12 mL) was mixed with 7.5mL of separation buffer, and the volume made to 29mL with water. After degassing, TEMED (30μL) and ammonium persulphate (1.0mL of a 5mg/mL solution) were added with mixing, and the solution immediately dispensed into gel tubes. Water or butanol was layered on top of the gels. After 30 minutes to allow for complete polymerization,

approximately 0.2mL of the stacking gel solution (1mL of acrylamide, 2.5mL of stacking gel buffer, 0.5mL of ammonium persulphate, and 15μL of TEMED and the volume made to 10mL with H_2O) was applied to the top of the separation gel.

Lyophilized samples were dissolved in the sample buffer with heating to 50°C for 30 minutes and Bromophenol Blue (10-20μL) was added to each solution. After layering samples under the running buffer, electrophoresis was performed with constant current initially (1mA/gel) until sample had entered the gel and then at 3mA/gel until the tracking dye had reached the 8.0 cm mark.

B.2.3. Scanning of polyacrylamide Gels

The polyacrylamide gels after staining were exhaustively destained as described, and then scanned at 560nm in 10% acetic acid in an automatic Gilford Linear Transporter on a Beckman DU Gilford Spectrophotometer. The scans were recorded on a Fisher Scientific 'Recordall' chart recorder. The relative mobilities of the stained peptides were calculated when necessary, by relating the distance traveled from the origin to the center of peak (peptide band) on the gel scan, to the distance travelled by the tracker dye. Quantitation of the relative areas of certain peptide bands was generally performed by multiplying the peak height by the width at half peak height. The apparent molecular weights (M_r) of the peptides were calculated from a standard plot of relative mobility versus the $\log_{10}$ of the molecular weight. To minimize any variation between runs, and between gels themselves, a standard plot was obtained on each gel of interest. For the

cyanogen bromide peptides, α_1CB7 (24,900), and α_1CB3 (13,480) were used to estimate the molecular weight of peptides from the α_1 chain, and α_2CB3,5 (60,540) and α_2CB4 (29,500) were used for peptides derived from the α_2 chain.

B.2.4. Analysis of the Gels for Radiolabelled Peptides

To detect radiolabelled peptides, polyacrylamide gels were frozen with solid CO_2, and sliced into 1mm sections on a Brinkmann Gel Slicer (Rexdale, Ont.). These sections were placed individually into glass scintillation vials, to which 500μL of 0.6N N.C.S. Tissue Solubilizer (Amersham/Searle) was added. After incubation overnight at 35°C, 15μL of glacial acetic acid was added to each vial, followed by 10mL Liquifluor solution [42mL of Liquifluor (New England Nuclear) mixed with 1 litre of toluene). After 24 hours in the dark, to allow chemiluminescence to decay, the radioactivity was measured.

B.3. Assays

B.3.1. Microbiuret Assay for Protein

The microbiuret method as described by Itzhaki and Gill (1964) was used for the estimation of collagen concentration. To an aliquot (50-500μL) of sample made up to 500μL with water, 250μL of the biuret reagent [30% (w/v)NaOH, 0.22% (w/v) copper sulphate ($CuSO_4.5H_2O$) was added with mixing. After 30 minutes at room temperature the optical density was measured using a Gilford spectrophotometer at $A_{300\ nm}$. Gelatin (0.5mg/mL), of known moisture content, which was heated to

60°C for 30 minutes and cooled to room temperature, was used to obtain a standard curve.

B.3.2. Folin-Ciocalteu Reagent for Protein

Lowry's method (1951) was used for the determination of the protein content of [^{14}C]-carboxymethylated transferrin substrate used for MMP3 assay. The reagents required were prepared as follows:

A] 5% (w/v) Na_2CO_3 in 0.25 M NaOH.

B] 1% $CuSO_4.5H_2O$.

C] 2% NaK tartarate.

D] Alkaline Copper Solution: Made fresh by mixing 250μL of B + 250μL of C in a 25mL cylinder. To this was added 20mL of A.

E] Folin-Ciocalteu reagent: 1mL of 2N solution from BDH was diluted to 8mL with water.

F] Albumin Standard: A stock solution of 1mg/mL of bovine serum albumin in water was made.

In clean glass tubes (10 x 75mm), 10μL to 30μL of the sample or the standard solution to give a range of concentrations (1.25μg to 25μg in the final assay volume) were taken in duplicate. The volume made to 500μL with water, 250μL of solution D added and left at room temperature for 10 minutes. To each was then added 250μL of solution E and again left at room temperature for 30 minutes. The color developed was measured at 740nm.

B.3.3. Assay for Azocoll Digesting Activity

Non-specific neutral proteinase activity was estimated as described by Pettigrew (1978). In 1.5mL micro-centrifuge tubes, approximately 3.0 mg insoluble Azocoll was mixed with an aliqout of enzyme sample (25-500 μL), and made up to 1.5 mL with buffer to a final concentration of 0.05 M-Tris-HCl, pH 7.5, 0.2 M-NaCl, 0.005M-$CaCl_2$ and 0.02% (w/v) sodium azide. The samples were incubated at 37°C, for 20-24 hours on an end-over-end shaker set at the slowest speed. The mixtures were then centrifuged at 12,000 x g, for 3 minutes, the supernatant removed and the optical density at 520nm measured in a 1cm pathlength cuvette. Trypsin was used as a positive control.

B.3.4. Assay for Azocasein Digesting Activity

The ability to digest azocasein was determined essentially as described by Sapolsky et.al., (1974). Azocasein was dissolved in assay buffer at a concentration of 1mg/mL, by heating to 50°C, for 30 minutes. Aliqouts containing 0.1mg of azocasein, were dispensed into 1.5mL micro-centrifuge tubes, and enzyme solution was added. The volumes were made up to 0.450mL with buffer (final concentration 0.05M-Tris/HCl, pH 7.5, 0.2M-NaCl, 0.005M-$CaCl_2$ and 0.02% (w/v) sodium azide). The reaction was carried out at 37°C for 20-24 hours. Trichloroacetic acid (25% w/v) was added to a final concentration of 5%, and the precipitate of undigested azocasein sedimented at 12,000 x g. The supernatants were withdrawn quantitatively, and dispensed into 1.5mL micro-centrifuge tubes.

Sodium hydroxide to a final concentration of 0.5M, (which increases the pH of the solution and enhances the color) was then added with shaking. The solution was again clarified by centrifugation. The optical density at 450nm was then measured. Trypsin was used as a positive control.

B.3.5. Collagenase Assay

Acid soluble type I collagen (ASC) purified from fetal bovine skin was dissolved in 0.1 M acetic acid at a concentration of 2mg/mL by stirring overnight at 4^0C (Scott & Goldberg, 1983). The dissolved ASC was dialyzed against 1 litre of 0.01 M acetic acid at 4^0C, for three days with daily changes of the dialysate. The protein concentration of the solution was determined by microbiuret and stored at -20°C.

The semiquantitative method of Berman et. al.(1973) was used. To the ASC solution in 0.01 M acetic acid was added an equal volume of buffer of double strength. The concentration was made to 1mg/mL containing in final concentration 0.05M-Tris/HCl, 0.2M-NaCl, 0.005M-$CaCl_2$ and 0.02% (w/v) sodium azide, pH=7.4. This solution was degassed while on ice and non-heparinized glass tubes (1.2mm x 75mm, Fisher brand^R) were partailly filled by capillary action. Tubes were sealed at one end with Critoseal^R, St.Louis U.S.A.) and at the other end with a removable short length of plastic tubing plugged with dental wax. The collagen was then left overnight at 37°C to gel. The top of the gel in each capillary was marked. Samples to be tested for collagenase activity, were first adjusted to

the same molarity of salt (0.2M-NaCl) as that of the collagen gel, and then applied (5μL/gel), with a Hamilton 10μL syringe, above the opaque collagen gels. The tubes were sealed and incubated at 37°C. The rate of clearing (mm/hr) was measured at various intervals, from 2 to 48 hours. In the samples where excess of collagenase activity was seen (dissolution of the entire gel in less than 5 hours), the experiment was repeated by diluting the sample with buffer.

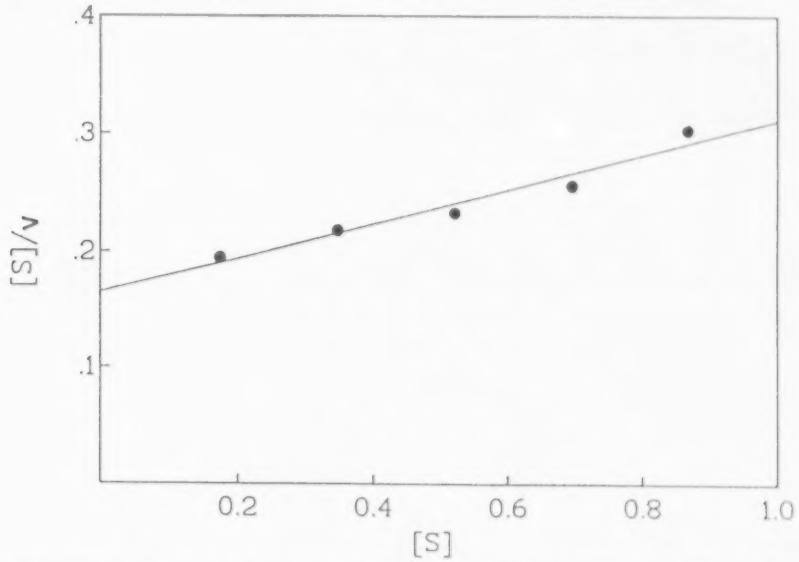


Fig 6. Determination of the K_m of collagenase for acid soluble collagen

For quantitative measurements the collagenase assay used was based on the conversion of α_1 to $\alpha_1 3/4$ peptides (modified from the protocol described by Sodek et. al., 1981). Acid soluble collagen was digested with the enzyme fractions, with or without inhibitor. Glucose (final concentration in assay of 0.18 gm/mL) was used to prevent gelling of the collagen. To determine the Michaelis constant, K_m (defined as the concentration of the substrate at which the velocity is half the

maximum value) for collagenase, assays were done at different concentrations of acid soluble collagen. The Michaelis and Menten (1913) equation is

$$v = V[S]/(K_m + [S])$$

where [S] is the substrate concentration, v is the velocity of the enzyme-catalyzed reaction and V is the maximum velocity. This equation can be rearranged to give

$$[S]/v = K_m/V + [S]/V$$

A plot of [S]/v versus [S] gives a straight line of slope 1/V and has an intercepts of K_m/V on the [S]/v axis and $-K_m$ on the [S] axis (Cornish-Bowden; 1976).

In experiments where inhibitors were dissolved in DMF, buffer containing the same amount of DMF was added to the enzyme and no enzyme control. The digested products were electrophoresed on 5% SDS polyacrylamide gels. The areas of these peptide peaks were measured, the $\alpha_1 3/4$ peptide value adjusted by a factor of 4/3 to account for loss of the $\alpha_1 1/4$, and the area representing α_1 chain summed. The ratio, expressed as a percent of $\alpha_1 3/4$ (adjusted) to the total area was related to the amount of collagen that was initially present in the incubation. The activity seen by the enzyme control was considered as 100% digestion during inhibition studies.

B.3.6. Gelatinase Assay

Assays to detect gelatinase activity were carried out using [^{14}C]gelatin prepared by heat denaturation (20 min at 52°C) of purified acetic-acid-soluble bovine type I collagen which had been labelled with [1-^{14}C]acetic anhydride (as

described earlier). The assay included 2 μg of substrate (3000 dpm) and 5-80 μL of enzyme preparation in a total volume of 100 μL buffer 1. After incubation at 37°C for 1 or 2 h an equal volume of 60% (w/v) trichloroacetic acid was added and the supernatant (150 μL) obtained after centrifugation at 1000 x g for 15 min was counted. The value for an enzyme-free control, which accounted for approximately 5% of total count of substrate, was subtracted from each count. The variation encountered in duplicate assays was less than 10%. A linear relationship between amount of enzyme and digestion was obtained using this method. One unit of gelatinase is defined as that amount of activity degrading 1 μg of substrate in 1 h in the above assay.

B.3.7. Telopeptidase Assay

In 1.5mL micro-centrifuge tubes, an aliqout (10 or 20μL) of ^{3}H-collagen ($NaB^{3}H_{4}$-reduced acid soluble fetal bovine skin collagen, purified by TCA and ethanol precipitations) was mixed with an equal volume of double strength assay buffer (0.10 M Tris/HCl, pH 7.5, containing 0.4 M-$NaCl_2$, 0.010 mM-$CaCl_2$ and 0.02% sodium azide). An aliquot (10-200μL) of the sample to be tested was added, and the solution made up to 240μL final volume with assay buffer, or with buffer that would normalize the concentration to that of the assay buffer constituents. The solutions were incubated at 20-23°C for a period that was dependent upon the estimated level of activity. This varied from 15-48 hours, but the majority of the incubations were for 20 hours. Concentrated formic acid (17μL)

and 50μL of bovine serum albumin (4mg/mL in 1.0N-formic acid), were added and the solution chromatographed on a column (0.7 x 4cm; 2mL total volume; Bio-Rad Laboratories), packed with Bio-Gel P10 (fractionation range 1,500-20,000), which was washed initially with 15mL of water and 7mL of 1.0N-formic acid. The columns were eluted with 1.0N-formic acid and the effluent collected in 7mL scintillation vials and counted after addition of 5mL of scintillation fluid, as described below. The five fractions that were collected were representative of the three regions of interest:

fraction#	Volume (mL)	
1	1.3	Vo fraction;
2	0.2	
3	0.2	
4	1.0	Vt fraction;
5	1.0	Vt fraction;

The large molecular weight peptides elute in fraction 1 whereas the small molecular weight fractions eluted in fraction 4 and 5. The levels of radioactivity in fractions 3, 4 and 5 were indicative of the presence of telopeptide- digesting activity in the assay. A histogram of a typical assay is shown in Fig 7.

Definition of units of telopeptidase activity: One unit is defined as that activity releasing all possible radioactivity from 1 μg of collagen in 1 h at 20°C.

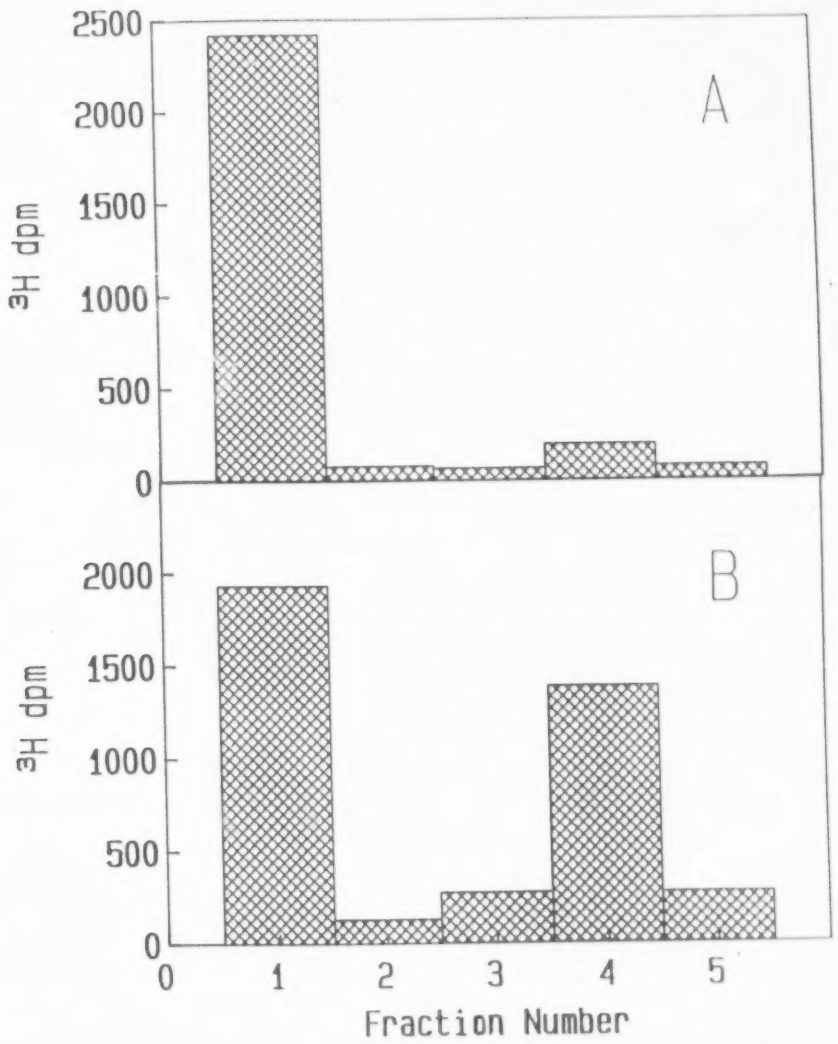


Fig 7. Histogram to show release of radioactive peptides on digestion of [^{3}H] labelled acid soluble collagen with the enzyme containing telopeptide digesting activity. The digest was chromatographed on Bio-Gel P10 (0.7 x 4 cm.) column with a fractionation range 1,500-20,000. Panel A is the control ASC and Panel B the ASC digested with telopeptidase.

Maximum Activity Curve

Several concentrations of denatured gelatin (0.5 mg/mL) solution were used to obtain a standard curve (μg of gelatin in 0.5 mL verses optical density at 300_{nm}) using the microbiuret assay (section B.3.1). Similarly 20 and 50 μL of ^{3}H ASC was used in duplicates in the microbiuret assay, and the absorbance read at 300_{nm}. These values were then used to calculate the concentration of the tritiated ASC. To determine the maximum activity that can be released by the telopeptidase activity, 20μL of collagen was used in the above telopeptidase assay with increasing amounts of explant medium (from 20 to 200 μL). The maximum dpm released were calculated from a plot of μL of enzyme verses dpm (Fig 8). Thus knowing the concentration in μg/ 20μL and the maximum dpm released by 20 μL, (dpm /μg) were calculated.

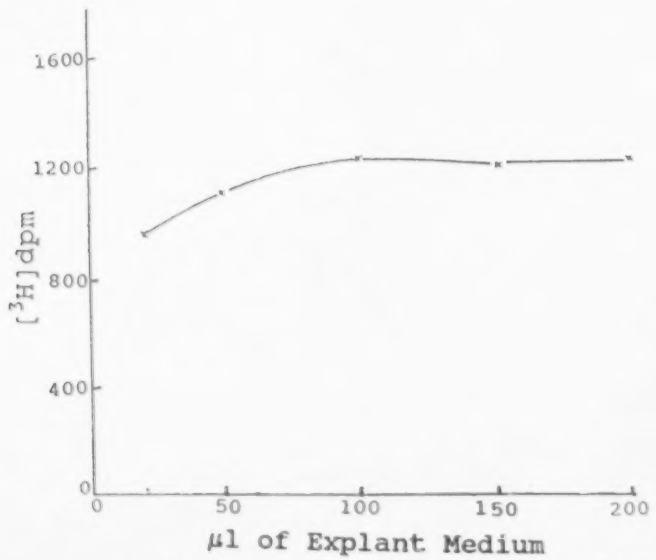


Fig 8. Maximum telopeptidase activity that can be released from 20 μL of ^{3}H-Sodium borohydride labelled acid soluble collagen using the telopeptidase assay and 20 - 200 μL of explant medium.

B.3.8. Stromelysin Assay

MMP-3 was assayed using a [^{14}C]-carboxymethylated transferrin (Cm Tf) substrate (Okada et al., 1986). Both MMP-3 and [^{14}C]-carboxymethylated transferrin were kindly provided by Dr. H. Nagase (University of Kansas Medical Center, Department of Biochemistry and Molecular Biology, Kansas City). The substrate concentration was determined by the method of Lowry (1951) using bovine serum albumin as a standard. In 600 μL micro-centrifuge tubes, 20 μL (80 μg) of [^{14}C] Cm Tf and 2 to 5 μL of MMP-3 (0.02-0.05 units of telopeptidase activity) were mixed. Inhibitor was added when inhibition studies were done. Total volume was made up to 50 μL with buffer (50 mM Tris/HCl, 20 mM NaCl, 10mM Ca^{2+} pH 7.6). A positive control was prepared using 4 μL of trypsin (containing 8 μg of trypsin). In experiments involving inhibition studies where the inhibitor was dissolved in DMF, buffer containing the same amount of DMF was added to the other controls. After incubation at 37°C for 30 mins. the digestion was stopped by adding 250 μL of 3% TCA and centrifuged at 1000 X g for 20 min on a bench top centrifuge. The supernatant (250 μL) was removed and counted after adding 5.0 mL of scintillation fluid.

B.3.9. Assay for Proteodermatan Sulphate Digesting Activity

Proteodermatan-sulphate-degrading activity was assayed using 10 to 40 μL of enzyme solution and 10 μg of bovine skin PDS, purified and characterized as described in Pearson et al. (1983), in a total volume of 30-50 μL of 0.125 M Tris-

0.125 M maleic acid-0.133 M NaCl-0.003 M $CaCl_2$-0.014% (w/v) NaN_3 adjusted to pH 7.4 with 5 M NaOH. Incubation was for 20 to 50 h, depending on the amount of activity present. Digestion was monitored by gel electrophoresis on 5% polyacrylamide gels containing 0.1% SDS (Scott et al. 1976). Control incubations with no enzyme were carried out at each pH. Densitometric scanning of gels stained with Coomassie Brilliant Blue R250 was used to determine the proportion of protein remaining undigested at the end of the incubation. One unit is defined as that amount of enzyme digesting 1 μg of PDS protein in 1 h at 37°C.

B.4. Other Techniques

B.4.1. Amino Acid Analysis

Samples were dissolved in 6N constant boiling HCl (Pierce Chemical Co.). When the peptides synthesized on the resin were to be analyzed, approximately 1 mg of the peptide-resin was taken in the hydrolysis tube. glacial acetic acid and HCl 0.5 mL (50:50 by volume) was added to this. Tubes were evacuated, sealed and incubated at 110°C, for 18-24 hours. The seal was then broken and samples dried in a dessicator containing NaOH pellets before amino acid analysis using Waters PicoTag method.

B.4.2. Liquid Scintillation Counting

Radioactivity was measured with a Philips Liquid Scintillation Counter (model PW4700). Aqueous samples were mixed with 5mL of Scintiverse™ (Fisher) in 7

mL vials or with 10mL of scintillation fluid in 20mL vials. When necessary, to avoid precipitation due to high salt concentrations, the sample was diluted with water (also see section B.2.4).

B.4.3. Cyanogen Bromide Digestion

The method used was as described by Scott & Veis (1976a). The samples to be digested, if in solution (less than 1 mg/mL of protein or peptide) were made up to 70% formic acid with the addition of an appropriate amount of concentrated formic acid. The solution was flushed with nitrogen for 2 minutes and cyanogen bromide to a final concentration of 12-20mg/mL was then added. If the sample was dry initially, cyanogen bromide in 70% formic acid was added, and the solution was briefly flushed with nitrogen. The digestion was carried out at 25-30°C for 4 hours with stirring. The digests were then diluted with a large excess of water and lyophilized. The samples were redissolved in water and lyophilized again to remove all traces of the cyanogen bromide and volatile reaction products. The products of digestion were analyzed by electrophoresis on 12% polyacrylamide gels.

B.4.4. Dialysis

Dialysis tubing (Spectrapor™, USA) with a 6,000-8,000 molecular weight cutoff was soaked and washed in distilled water, and then boiled in water for 15 minutes. The tubing was cooled, rinsed extensively with distilled water and used. All dialysis

was performed at 4°C, and involved 2-4 changes of the dialysis solution, each being of 24 hour duration.

B.5. Synthesis of Peptide Substrates and Inhibitors

B.5.1. Purification of Solvents

Molecular seives: These were dried in a muffle furnance at 400°C for 3 hours, cooled and kept in a desicator under vacuum until used.

Triethylamine: (b.p. = 89.4°C). Triethylamine was dried over Linde type 4A molecular sieve. In a 500mL round bottomed flask 250mL of the dry TEA was taken and purified by fractional distillation and then stored in a brown bottle.

1,4-Dioxane: (b.p. = 101.3°C). In a 1 litre round-bottomed flask 800mL of 1,4-dioxane was taken and potassium hydroxide pellets added. This was refluxed until fresh addition of KOH gave no more resin formation (brownish coloured due to the acetaldehyde). This was then filtered through paper, refluxed again over sodium until the surface of the metal was not further discoloured and finally distilled over sodium. The purified 1,4-dioxane was stored over Linde type 4A molecular sieve.

Isopropanol: (b.p. = 82.5°C). 1 litre of isopropanol was refluxed over 200 gms. of anhydrous calcium oxide for 2 hours, distilled and stored over molecular sieves.

B.5.2. Hydrogenation

In a three necked round bottomed flask (sizes from 10 - 100 mL according to

the volume of the reactants) the compound (Benzyloxycarbonyl protected peptide or compound #9), was taken along with catalyst (5% palladium on charcoal 10 - 100mg). To this 0.5mL -2.0mL of dry methanol was added. The round bottomed flask was connected on one side to a lecture size hydrogen gas cylinder (Matheson) and on the other to a mercury monitor (to monitor positive flow). A steady flow of hydrogen was passed through the reaction mixture. The progress of the reaction was monitored by TLC.

After the reaction was completed (1 hour), the catalyst was filtered. To the filtrate decolourizing charcoal was added and the suspension filtered again. The solvent was evaporated on a rotary evaporator.

B.5.3. Cleaning of the Chloromethyl Resin

Merrifield resin (10 gm) was taken in a 1 litre round-bottomed flask, and washed with 100 mL of tetrahydrofuran (THF) [100 mL at a time], with gentle shaking. The fines and THF were removed with a scintered glass rod attached to a vacuum system.

The washings were done as below, repeating each washing several times, in order to remove any free chlorides and alkali left during the manufacturing procedure and finally to remove the excess acid.

1. 100 mL of THF 4 times
2. 100 mL of THF:H_2O (75:25, v/v) 6 times
3. 100 mL of THF:3N HCl (75:25, v/v) 6 times
4. 100 mL of THF:H_2O (75:25, v/v) 8 times
5. 100 mL of THF 5 times

When no more fines were seen, 600 mL of methanol was added and it was filtered through a sintered funnel. The resin was then dried over P_2O_5 in a vacuum desicator.

B.5.4. Preparation of the Cesium Salt of Glycine

Boc-glycine (3.15 gm/18mM) was weighed in a 500 mL round bottomed flask and dissolved in 10 mL of a 3:1 mixture of ethanol and water. To this was added with shaking a solution of cesium bicarbonate 3.9 gm (18 mmoles + 10%) in 10 mL of water. The pH was checked to be between 6 and 7. After the effervesence had stopped, the solution was evaporated to dryness on a rotary evaporater.

B.5.5. Coupling of Boc-Glycine Cesium Salt to the Resin (Method of Gisin)

The cleaned resin (10 gm/9 mMoles) was added to the dry Boc-glycine cesium salt in the round bottom flask. 60 mL of dimethylformamide (DMF) was added, a little at a time. The reaction was stirred gently, while in a 50°C water bath with a motor operated rod stirrer, for 24 hours. The resin washed several times (to remove unreacted cesium salt) with DMF, DMF:H_2O (1:1), a mixture of 9:1 DMF and water, again with DMF and finally with ethanol. The resin was stored in a vacuum desicator over P_2O_5.

B.5.6. Symmetric Anhydride of Boc-amino acid

To make the symmetric anhydride, 4 equivalents of amino acid and 2

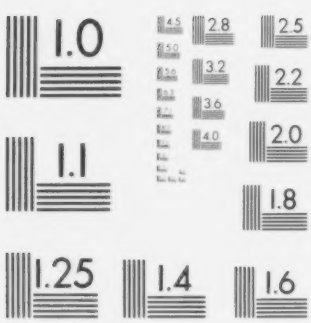
equivalents of DCC were used. These reagents were weighed into small vials and dissolved separately in DCM. The DCC solution was then added to the amino acid and the reaction is allowed to go for half an hour in the cold room. This procedure was not used for Boc-Arg(Tos) and Boc-Gln since it has been reported to cause double insertion of arginine residues into the peptide and dehydration of the amides.

B.5.7. N-hydroxybenzotriazole (HOBt) active ester

Five equivalents of the amino acid and 7.5 equivalents of HOBt were dissolved in 3 mL of DMF. In a separate vial 5.5 equivalent of DCC were dissolved in 1 mL of DMF. Both the vials were cooled to 0°C. The DCC solution was slowly added to the amino acid mixture using 1 mL of DMF to transfer. This mixture was then stirred for 1 min and allowed to stand at room temperature for 30 min. The resin was rinsed twice with DMF so it was in the correct solvent for coupling. The reaction mixture was transferred to the reaction vessel by filtering the mixture through a pasteur pipet with glass wool as a filter. One mL of DMF was used to transfer and the reaction mixture was stirred for 30 min to 1 h. The resin was then rinsed several times with DMF before proceeding to the next step.

B.5.8. Synthesis of Peptide Substrates

ThepeptideN-acetyl-Gly^{-3}-Pro^{-2}-Pro^{-1}-Ser^{1}-Gly^{2}-Gly^{3}-Tyr^{4}-Asp^{5}-Leu^{6}-Ser^{7}-Phe^{8}-Leu^{9}-Pro^{10}-Gln^{11}-Pro^{12}-Pro^{13}-Gln^{14}-Glu^{15}-Lys^{16}-Ala^{17}-His^{18}-Asp^{19}-Gly^{20}-Gly^{21}-Arg^{22}-



MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

Tyr^{23}-Tyr^{24}-NH_2 was synthesized by the Alberta Peptide Institute (Department of Biochemistry, University of Alberta) and supplied as a crude product, which was purified by gel filtration on a 1 cm x 65 cm column of Sephadex G-50 Superfine, eluted with 0.1% (v/v) trifluoroacetic acid. Purity was assessed from the amino acid composition and by chromatography on a C_{18} reversed-phase HPLC column Vydac 201TP104. These methods are described in detail by Scott, P.G. (1986), in a report of a spectroscopic study on the C-telopeptide isolated from type I collagen. Bacterial collagenase was used to prepare the material for that study and hence it included at its N-terminal end the final Gly-Pro-Pro tripeptide from the triple-helical domain of the α-1 chain. This tripeptide was included in the synthetic material. N-acetyl-Pro-Gln-Pro-Pro-Gln-Glu-Lys-Ala-His-Asp-Gly-Gly-Arg-Tyr-Tyr-NH_2, N-acetyl-Gly-Pro-Pro-Ser-Gly-Gly-Tyr-Asp-Leu-Ser-Phe-Leu-NH_2 and N-acetyl-Pro-Gln-Pro-Pro-Gln-Glu-COOH, were synthesized on Beckman model 990 peptide synthesizer using Merrifield resin(to give C-terminal acid) or benzhydrylamine resin (to give the C-terminal amide). All α amino groups of the amino acids used for synthesis were protected with the Boc group. The side chains were also protected. Coupling of the amino acid was done by dicyclohexylcarbodiimide activation or by symmetric anhydride. Completion of the coupling was tested with picrate or ninhydrin (Stewart and Young, 1984; pg. 105-107). The peptides were cleaved off the resin with hydrogen fluoride (Stewart and Young, 1984; pg. 38-40) and purified on RP-HPLC (see methods section B.5.11). Ac-PQPPQEKAHDGGRYY-NH_2 was synthesized to include Tyr in order to be able to

monitor at 280 nm.

B.5.9. Hydroxamate Derivatives of Peptides

(a) Synthesis of HONH-Bzm-Ala-Gly-NH_2

This compound was synthesized by modifications to the method of Nishino and Powers (1978) and is described below (also see scheme 1). The esters diethyl benzyl malonate, potassium ethyl benzyl malonate and monoethyl benzyl malonate that were intermediates in the synthesis were tested as follows. To two drops or a few crystals of the compound taken in a test tube, was added 1 mL of 0.5 N hydroxylamine hydrochloride in ethanol. To this was then added 0.2 mL of 6N aqueous NaOH and the mixture was heated to boiling. After cooling slightly, 2 mL of 1N HCl was added. Finally when a few drops of 5% ferric chloride solution was added, a distinct burgundy color or precipitate indicated a positive test for ester.

Glycine benzyl ester p-toluene sulfonate (1)

To glycine (10 gm, 133 mmol) and p-toluene sulfonic acid monohydrate (26.22 gm, 138 mmol) was added 60 mL of freshly distilled benzyl alcohol and 100 mL of toluene. The mixture was refluxed connected to a Dean Stark apparatus. When no more water was formed the refluxing was stopped, the mixture cooled to room temperature, and diluted with 250 mL of ether. On further cooling crystalline glycine benzyl ester p-sulfonate was formed. This was recrystallized from

methanol: ether. Yield=40 gm (90%); mp 133-136°C R_f^1 = 0.69.

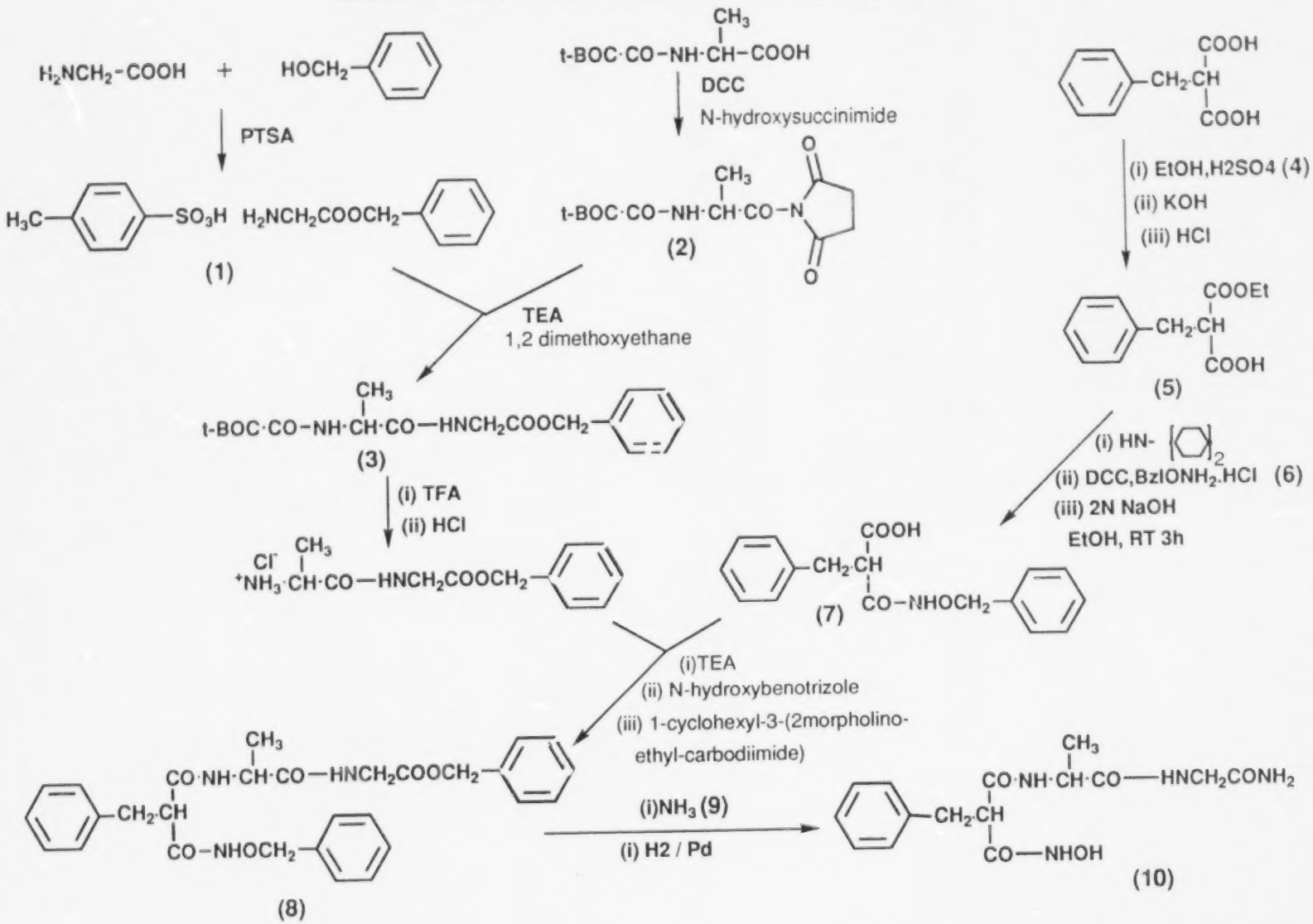
N-Hydroxysuccinimide ester of t.Boc.alanine (2)

To a chilled solution of t-Boc-L-Ala (5 gm, 0.027 mmol) and N-hydroxysuccinimide (3.1 gm, 0.027 mmol) in dry 1,2, dimethoxymethane (100 mL) was added dicyclohexylcarbodiimide (5.5 gm, 0.027mmol). The mixture was stirred overnight in the cold. The separated N,N'-dicyclohexylurea was filtered and the solvent evaporated. The crude product was recrystallized twice from isopropanol. Yield= 6.0 gm,(80%); mp=143-147°C.

tert.Butyloxycarbonyl-L-alanine-glycine benzyl ester (3)

To Glycine benzyl ester p-toluene sulfonate (6.4 gm, 18.8 mmol), tertbutyloxycarbonyl-L-Alanine N-hydroxysuccinimide ester (5.4 gm, 18.8 mmol) and triethylamine (2.6 mL=1.9 gm, 18.8 mmol) was added 90 mL of dry 1,2-dimethoxyethane. The mixture was stirred at room temperature for 30 - 60 minutes, then poured into ice-cold water (150 mL) with stirring. The material which separated was allowed to solidify. The crude product was washed with water, dried and recrystallized from ethanol and water. Yield= 5.34 gm (85%); mp=84-86°C R_f^1 0.85.

Scheme 1. Synthesis of HONH Bzm- Ala-Gly-NH2.



Diethyl benzyl malonate (4)

Benzyl malonate (10 gm, 0.052 mmol) was refluxed with 100 mL of ethanol and a few drops of concentrated H_2SO_4 for approximately 30 hrs. Completion of the reaction was measured by thin layer chromatography in methanol:chloroform 1:4. R_f=0.88. The solvent was concentrated and the liquid was taken in dichloromethane and washed with 4% $NaHCO_3$ and water. The organic layer was then dried over magnesium sulfate and concentrated to give pure diethyl benzyl malonic acid. Yield = 12.0 gm (93%). This step was repeated several times.

Monoethyl benzyl malonate (5)

To diethyl benzyl malonate 4 (29 gm, 0.116 mmol) was added 38 mL of ethanol. To the above while stirring at room temperature was added 6.80 gm of KOH in 100 mL ethanol over a period of 1 hour. The mixture was then stirred for an additional 2 hours and left standing overnight. The solvent was evaporated to give a precipitate of potassium ethyl benzyl malonate. This precipitate was taken in water and the unreacted ester was extracted with ether. The aqeous layer was kept on ice and 10 mL of concentrated HCl was slowly added with stirring. The monoester formed was extracted in ether. Yield = 22.15 gm.

Monoethyl benzyl malonic acid dicyclohexylamine salt

Ethyl 2-(N-benzyloxycarboxamido)-3-phenylpropanoate (BzlONH-Bzm-OEt) (6)

To a chilled solution of monoethyl benzylmalonic acid dicyclohexylamine salt

(10.15 g, 25 mmol) and $BzlONH_2$.HCl (3.99 gm, 25 mmol) in dioxane (50 mL) was added N,N'-dicyclohexylcarbodiimide (5.03 gm, 25 mmol). The mixture was stirred overnight at room temperature, then filtered from N,N'-dicyclohexylurea. The residual oil remaining after evaporation was dissolved in ethyl acetate and washed successively with 2% HCl, 4% $NaHCO_3$, and water. After drying over $MgSO_4$ the ethyl acetate solution was evaporated to give an oil. Yield = 9.37 gm.

2-(N-Benzyloxycarboxamido)-3-phenylpropanoic acid (BzlONH-Bzm-OH) (7).

To a solution of 6 (9.37 gm) in ethanol (75 mL) was added 2 N NaOH (75 mL). The mixture was allowed to stand at room temperature for 3 hour, then acidified with 6 N HCl to pH 3. The evaporation of ethanol and addition of water gave a crystalline product, which was recrystallized from ethanol-water. Yield 1.73 gm, mp = 159°C.

2-(N-Benzyloxycarboxamido)-3-phenylpropanoyl-L-alanylglycine benzyl ester (BzlONH-Bzm-L-Ala-Gly-OBzl) (8).

To a chilled solution of 7 (1.0 gm, 3.35 mmol), H-L-Ala-Gly-OBzl.HCl which was added 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate (1.42 gm, mmol)The reaction mixture was stirred overnight at room temperature, then evaporated. To the residual syrup was added 2% HCl. The crystalline precipitate was collected on a filter paper and washed with 4% $NaHCO_3$ and water. The crude product was recrystallized from ethanol-ether. Yield = 135 mg.

2-(N-Benzyloxycarboxamido)-3-phenylpropanoyl-L-alanylglycinamide (BzlONH-Bzm-L-Ala-Gly-NH_2 (9)

The benzyl ester 8 (200 mg), from step 8 repeated twice, was dissolved in methanol saturated with ammonia at 0°C (20 mL). The mixture was allowed to stand for 24 hour in tightly stoppered vessel. The residual solid obtained after evaporation was recrystalized from methanol-ether. Yield = 88.5 mg.

2-(N-Hydroxycarboxyamido)-3-phenylpropanoyl-L-alanylglycinamide (HONH-Bzm-L-Ala-Gly-NH_2 (10)

The compound 9 (80.0 mg) was hydrogenated (see section B.5.3.) in the presence of Pd-C in methanol-acetic acid-water (4:5:1, v/v) (10mL). After removal of catalyst, the solution was evaporated to the dryness. The resulting solid was crystallized from 95% ethanol-ether. Yield =40 mg.

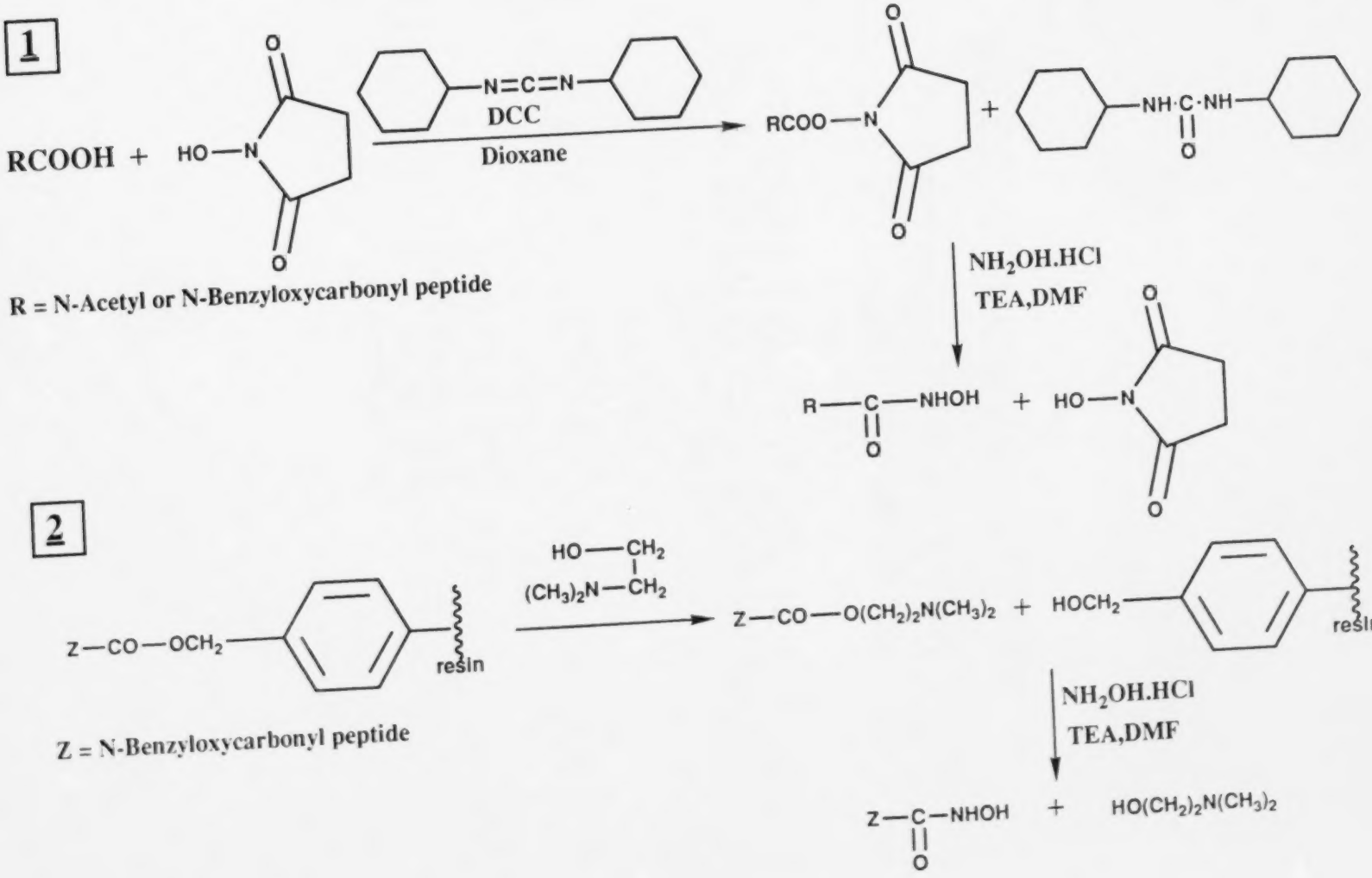
(b) Synthesis of peptide Hydroxamates

Z-Gly-Leu, Z-Leu-Gly and Z-Pro-Leu-Gly were obtained as the free acids from Sigma Chemical Co., (St. Louis, MO.). Z-Pro-Pro-Leu-Gly, Z-(D)Pro-Pro-Leu-Gly and Z-Pro-Gly-Gly were obtained as the peptide resins (Multiple Peptide Systems, San Diego, CA, U.S.A.). Z-Gly-Pro-Leu-Gly, Z-Leu-Pro-Leu-Gly and N-acetyl-Pro-Leu-Gly were synthesized in the Department of Biochemistry, University of Alberta, on a Beckman model 990 synthesizer using standard procedures (Erickson and Merrifield, 1976). The first (glycine) residue was added as the cesium salt (Gisin

et al., 1973).

Peptide hydroxamates were synthesized using one of the two methods described below depending, on whether the peptide was on the resin or as a free acid (scheme 2). Peptides with the N-benzyloxycarbonyl (Z) group intact were cleaved from the resin by transesterification using a modification of the method of Bodansky (Bodansky, 1984). Peptidyl resin (0.5 - 1 g) was washed several times with DMF and then suspended in 20 - 40 mL of dry DMF and an equal amount of dimethylaminoethanol. The suspension is stirred for about 24 hours, filtered and the resin washed with DMF (three times, 20 mL each time). The filtrate and washings were pooled and concentrated to approximately 2 mL on a rotary evaporator at room temperature. Assuming 100% cleavage of the peptide from the resin as the dimethylaminoethyl ester, equimolar amounts of hydroxylamine hydrochloride and triethylamine were dissolved in dry DMF, filtered and the filtrate added to the peptide ester. The mixture was stirred overnight at room temperature and evaporated to a residual syrup which was taken up in ethyl acetate and washed successively with 0.2 N HCl, 4% (w/v) $NaHCO_3$ and water. The organic layer was dried over anhydrous $MgSO_4$, filtered and evaporated to dryness.

Scheme 2 Preparation of peptide hydroxamate



The peptides that were obtained as free acids (Z-Gly-Leu, Z-Leu-Gly, Z-Pro-Leu-Gly and NAc-Pro-Leu-Gly) were converted to active esters using an equimolar amount of N-hydroxysuccinimide and N,N'-dicyclohexylcarbodimide in dioxane (Nishino and Powers, 1978). The N,N'-dicyclohexylurea was filtered off and the filtrate evaporated. These peptide esters were then converted into the corresponding hydroxamates as described above.

The N-(benzyloxycarbonyl) group was removed from portions of certain peptides by catalytic hydrogenation using a palladium/ charcoal catalyst (House, 1972). Reaction progress was monitored by TLC on Silica-Gel G plates (Merck), developed with 10% (v/v) CH_3OH in $CHCl_3$ or butanol:acetic acid:pyridine:water (4:1:1:2). Hydroxamic acids were detected by a colorimetric reaction with 5% $FeCl_3$ in 6% acetic acid.

B.5.10. Peptide Synthesis by Fmoc-Polyamide Synthesis

The peptide amides and acids were synthesized by Fmoc synthesis on the LKB Biolynx™ 4175 Fmoc-polyamide synthesizer. Fmoc-Ultrosyn C resin was used for the synthesis of amides and the synthesized peptide was cleaved off the resin by 95% TFA. Fmoc-Ultrosyn A resin was used for peptide acid synthesis. The resins and the amino acids were from Pharmacia LKB Biochrom Ltd., Cambridge, England. The Fmoc amino acids were purchased as pentafluorophenyl esters. DMF and dichloromethane were purchased from Alberta Peptide Institute, Edmonton, Alberta. The side chains of Asp and Glu were protected by tboc

group. The last proline was coupled as Z-Pro-succinimide or Z-pro-p-nitrophenyl ester purchased from Sigma Chemical Co., (St.Louis, MO.). After cleavage from the resin the peptides were purified on RP-HPLC as described below.

B.5.11. Purification of peptide substrates and inhibitors

Reversed-phase HPLC on Vydac C_{18} RP-218-TP.1010 columns 1 x 25 cm. was used for purification of peptide substrates, peptide hydroxamates and some peptide acids. In general, an isocratic gradient of 0.1% TFA in water (eluent A), was run for 10 min followed by a linear gradient of 1% B/min, where eluent B was 0.1% TFA in acetonitrile. A flow of 2 mL/min was used and 1 min fractions were collected. The peptide elution was monitored at an absorbance of 214nm. Aliquots of the fractions under the peaks were individually dried and analyzed. Similarly aliqouts of the fractions thought to contain the peptide hydroxamates were dried and spotted on Merck Silica TLC plates (G60, F254) They were developed with 20% methanol in chloroform and sprayed with 5% (w/v) $FeCl_3$/6% (w/v) acetic acid in ethanol. Fractions containing the required peptide or peptide hydroxamates were pooled and dried. The purity of each peptide and peptide hydroxamate was checked by amino acid analysis and proton NMR either on a 300 MHz Bruker AM 300 in the Department of Chemistry or on a 360 MHz Bruker in the Department of Pharmacy, University of Alberta.

B.6. Purification of Human Gingival Fibroblast Telopeptidase

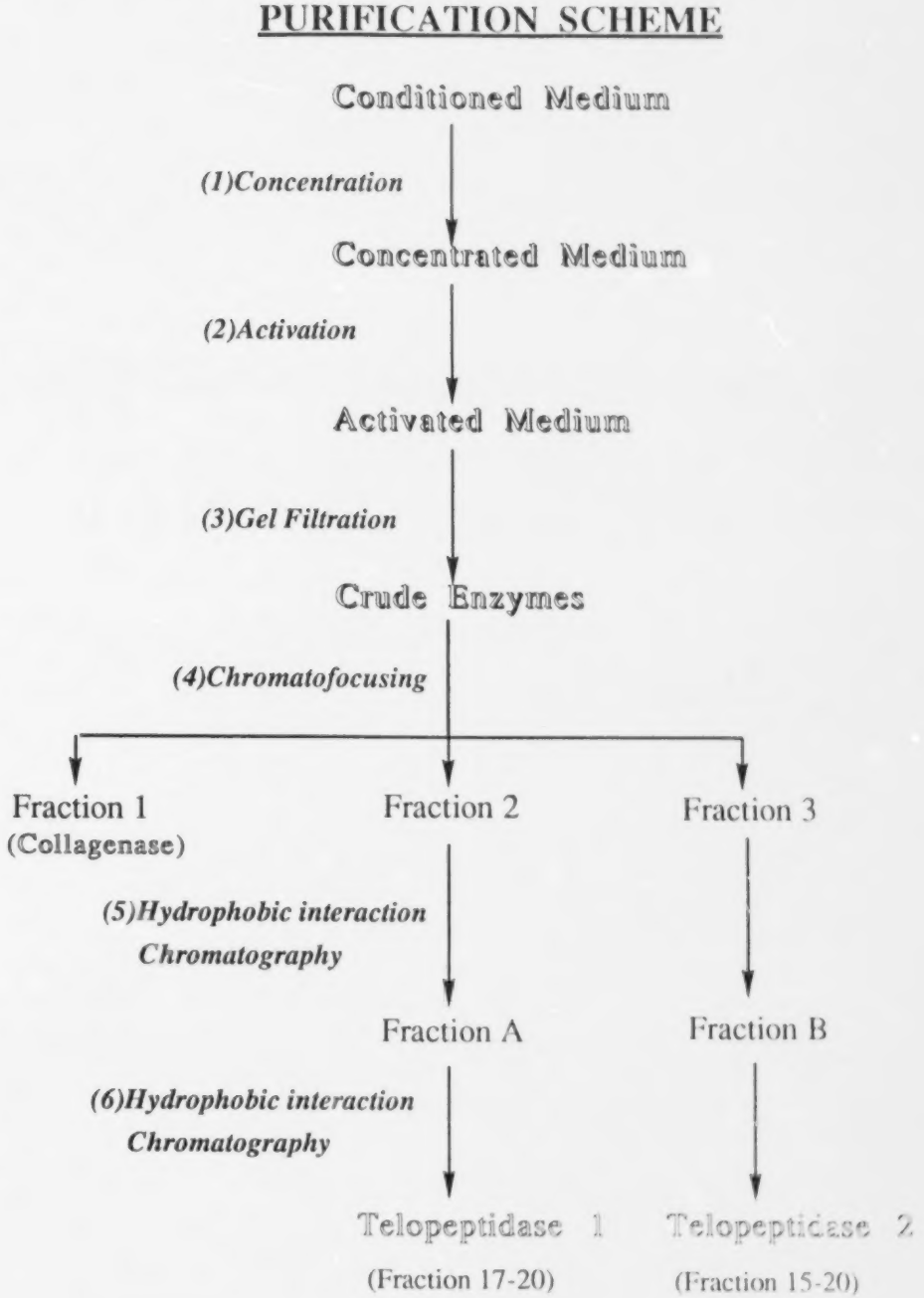
The purification steps were as outlined in scheme 3. Human gingival fibroblasts were cultured and stimulated to secrete proteinases by concanavalin A, as described in Nakano & Scott (1986). The medium was changed daily for 3 days and stored frozen at -22°C after addition of sodium azide (0.02% w/v). All procedures except the activation of the medium were done at 4°C.

B.6.1. Concentration of the Conditioned Medium

Gingival fibroblast culture medium (1L) was thawed and concentrated to 60 mL using a Minitan™ ultrafiltration system with a 10,000 MW cutoff (Millipore Corp.). To 60 mL of concentrated medium 12.0 mg of NaN_3 (0.02%) was added.

B.6.2. Activation of the Concentrated Medium

Trypsin bound covalently to agarose beads was used to activate the proteinases. An aliquot of the trypsin-agarose (50 units) was washed 3 times with assay buffer in a screw capped 15 mL culture tube, to this was added 15 mL of the concentrated medium. This was left at room temperature (22°C) for up to 2 hrs, gently shaking on an end over end shaker, to resuspend the trypsin agarose. The mixture was centrifuged, and the supernant containing active enzymes removed.



Scheme 3. Purification scheme for human gingival fibroblast telopeptidase. (See results for details.)

B.6.3. Gel Filtration Chromatograghy

This medium was then applied to a column of Bio-Gel agarose A0.5m (5cm x 99cm) equilibrated and eluted with 0.05MTris-HCl-0.005M$CaCl_2$-0.2M$NaCl_2$-0.05%Brij 35-0.02%NaN_3, pH 7.4 (Buffer 1). Fractions (10.4 mL) each were collected. The column was run at 4°C. The column was calibrated for molecular weight estimation by the chromatography of protein standards with ^3H_2O to determine total column volume. Every third fraction was assayed for gelatinase activity (90 μL/fraction at 37°C for 1 hr). Similarly 100 μL of every third fraction was used to test for the nonspecific proteinases azocoll and azocasein. In order to test for the presence of telopeptidase and collagenase activity, 25 μL were pooled from 4 tubes in a row (total of 100 μL). From this pool 5 μL was used for collagenase (at 37°C for 16.5 hr) and 50 μL for telopeptidase at (21°C for 20 hrs).

B.6.4. Concentration and Desalting

Fractions containing the telopeptidase activity from the gel filtration column (fractions 100 to 130) were pooled and concentrated approximately 8 fold to 16 mL using immersible membrane ultrafilters with a 10000 M_r cutoff (CX-10; Millipore). The buffer was then exchanged for 0.025M Tris-HCl-0.005M $CaCl_2$-0.05% Brij 35-0.03 % toluene (pH 7.4) using prepacked 1.5 x 5 cm colums of Sephadex G-25 (PD-10; Pharmacia). Two mL of the sample was loaded on each column. The first 2.5 mL that eluted was discarded and the next 3 mL collected for further purification. The PD-10 column was reused after washing off the salt with

several volumes of buffer. The above four steps were repeated with another 1 litre of explant medium and the final fractions were pooled together.

B.6.5. Chromatofocusing

The above pooled sample was then applied to a PBE 94™ chromatofocusing column previously equilibrated with 0.025 M imidazole-0.05%Brij 35 (pH 7.4). The column was eluted with 340 mL of Polybuffer 74 (pH 3.9) at a flow rate of 35 mL/h and fractions (4.5 mL) collected into tubes containing 0.5 mL of 2MNaCl-0.05M$CaCl_2$-0.2%NaN_3. The pH of each was adjusted to 7.4 with 0.5M Tris within 0.5 hrs of elution. Collagenase, telopeptidase and gelatinase assays were performed.

B.6.6. Hydrophobic Interaction Chromatography

The two main peaks containing telopeptidase activity were each pooled and treated separately (see results). Fractions 36 to 58 (118 mL, fraction 2) were pooled, 12.98 gm $(NH_4)_2SO_4$ was added to give 20 % saturation and this solution was then applied to a 5 x 1.5 cm column of Phenyl-Sepharose CL-4B™ equilibrated in buffer 1 (see methods section B.6.3), containing ammonium sulphate (to 20% saturation), but without Brij 35. Elution was performed with a gradient formed from 15 mL of the above buffer and 15 mL of 50% (v/v) ethylene glycol in buffer without ammonium sulphate (limit buffer). Similarly fractions 72 to 87 (fraction 3) off chromatofocusing were pooled (75 mL), saturated with NH_4SO_4

and applied to Phenyl-Sepharose.

Hydrophobic interaction chromatography was repeated on the fractions found to contain telopeptidase, without ammonium sulphate. Fractions containing telopeptidase activity were pooled, concentrated with CX-10 ultrafilters, passed down a PD-10 column equilibrated with buffer 1 to remove ethylene glycol, and reapplied to Phenyl-Sepharose equilibrated and eluted with buffer 1. Elution was performed with a gradient formed from 15 mL of buffer 1 and 15 mL of 50% (v/v) ethylene glycol in buffer 1 (limit buffer).

B.6.7. Optimum pH Study for Proteodermatan Sulphate Digesting Activity of Telopeptidase 2

Nakano & Scott (1988) have shown the presence of two proteodermatan sulphate-degrading proteinases produced by human gingival fibroblasts, one with an acidic pH optimum (6.0) & another with neutral pH optimum (7.0). This experiment was done in order to check if telopeptidase 2 was similar to the acidic proteinase seen by them. 10 μL of telopeptidase 2, 10 μL of bovine skin proteodermatan sulphate and 12 μL of buffer (0.125M Tris-0.125M maleic acid-0.133M NaCl-0.033M $CaCl_2$-0.014%(w/v) NaN_3 were mixed and adjusted to the required pH with 5 M NaOH. Incubations were carried out in duplicates at 37°C for 14.5 h. Control incubations with no enzyme were carried out at each pH. Digestion was monitored by electrophoresis on 5% polyacrylamide gels containing 0.1% SDS. Densitometric scanning of gels stained with Coomassie Brilliant Blue

R250 was used to determine the proportion of protein remaining undigested at the end of the incubation. One unit is defined as that amount of enzyme digesting 1 μg of PDS protein in 1 h at 37°C.

B.7. Large Scale Incubations with Acid Soluble Collagen

B.7.1. Large Scale Incubation of Collagen with Telopeptidase 1 and 2

Tritiated soluble collagen (360 μg in 0.675 mL of 0.01 M acetic acid) was taken in separate tubes. The pH of each was adjusted to 7.4 with 0.5 M Trizma. To one tube was added 608 μL (0.9 U by telopeptidase assay) of telopeptidase 1 (see Scheme 3 in section B.6.) to the other tube was added 549 μL (0.9 U by telopeptidase assay) of telopeptidase 2 (see Scheme 3 in section B.6.). This was incubated at 20°C for 72 hrs. The incubation mixture was then dialyzed against 0.1M acetic acid at 4°C for 3 days with daily changes. The samples were then lyophilized. In order to test for the presence of collagenase, 50 μg of each sample was run in duplicate on 5% polyacrylamide gels containing 0.1% SDS as described in (Scott et al 1976). A sample (240 μg) of each sample was weighed into a clean screwcapped tube and cyanogen bromide digestion was done as described (section B.4.3).

B.7.2. Large Scale Incubation of Collagen with Purified and Recombinant Stromelysin

Enzyme (stromelysin/MMP-3) preparations were incubated with radioactive

or non-radioactive acetic acid soluble collagen. Aliquots of collagen (80-160μg/mL final concentration, unless otherwise indicated) were incubated with the proteinase (purified or recombinant MMP3 corresponding to approximately 1 unit of the telopeptide cleaving activity using the tritium labelled acid soluble collagen). The procedure was as described for telopeptidase (see above).

B.8. Determination of Scissile Bonds for Telopeptidase

The general approach was that the peptide substrate (2.4 - 180 nmoles) dissolved in buffer 1 was incubated with enzyme (0.15 to 2.0 telopeptidase units determined by the telopeptidase assay). Incubation was for 17 to 80 hrs. at 20 to 22°C. Controls were made with buffer but no enzyme. The pH of each sample was adjusted to that of the eluent and then run on RP-HPLC using different ion-pairing agents or on strong cation exchange chromatography (chromatography conditions for each described below). Fractions corresponding to all the peaks were pooled, dried and analyzed.

B.8.1. Action of Explant Culture Medium on the Entire Synthetic α_1C-telopeptide

The sequence of the C-telopeptide (α_1C-telo-1) synthesized by API is as follows: N-CH_3CO-GPPSGGYDLSFLPQPPQEKAHDGGRYY-NH_2

Using this peptide several attempts were made to locate the scissile bond(s) for telopeptidase present in unfractionated explant medium. The peptide (10 μg/2.4

nmoles) was dissolved in 230 μL of buffer 1. To this was added 10 μL of the active explant medium and incubated at 21°C for 18 hrs.

B.8.2. Action of Purified Telopeptidase on the Entire α_1C-telopeptide

The synthetic peptide α_1C-telo-1 (116 μg/34 nmoles) was dissolved in 40 μL of buffer 1. To this was added 300 μL (corresponding to 1.4 units by the telopeptidase assay) of telopeptidase 2 (See Scheme 3 in section B.6). Incubation was for 68 hrs at 20°C.

B.8.3. Action of Purified Telopeptidase on Ac-PQPPQEKAHDGGRYY-NH_2 (α_1C-telo-2)

To 100 μL (0.18 μmoles) of α_1C-telo-2 (3 μg/ μL in buffer 1), 400 μL of telopeptidase 2 (see Scheme 3 in section B.6) was added. The total volume was made up to 600 μL with buffer 1. Incubation was at 20°C for 53 h. The digest was loaded on RP-HPLC using trifluoroacetic acid as ion-pairing agent. The above control and digest were also run on a Synchropak S-300 (strong cation exchange) column. The buffers used were A = 5 mM KH_2PO_4 pH 6.5. Buffer B = 5 mM KH_2PO_4, 0.5 M NaCl pH 6.5. A flow of 1 mL/ min and a gradient of 2% B/ min was used. The main peaks were dried and rerun on RP-HPLC. The fractions under the peaks were dried and analyzed. Separation of the digested fragments was also done using heptafluorobutyric acid (see results section C.3.3) as a hydrophobic ion-pairing agent on RP-HPLC. The elution was done using the buffer

system 0.1% Aq HFBA and 0.1% HFBA in acetonitrile.

B.8.4. Action of Purified Telopeptidase on Ac-GPPSGGYDLSFL-NH_2 ($N\alpha_1$C-telo)

Peptide substrate $N\alpha_1$C-telo (14.6 nmoles) was digested with 0.065 units of purified telopeptidase 2 at 20°C for 64.5 h. The digested fragments were separated by reversed phase HPLC.

B.9. Determination of the Scissile Bonds for Stromelysin on α_1CTELO-2 & $N\alpha_1$C-TELO

$N\alpha_1$Ctelo (8.32 nmoles) was incubated with 3 μL of purified stromelysin / MMP-3 (0.15 units of telopeptidase) and the total volume was made to 100 μL with buffer 1. This was incubated at 20°C for 80 hrs. and then run on RP-HPLC The run conditions were similar to those used for telopeptidase (see Fig 19).

Similarly 6.5 nmoles of α_1C-telo-2 peptide was incubated with 0.55 μg of MMP-3 (0.03 units of telopeptidase). The volume made up to 80 μL with buffer A, and incubation was at 20°C for 53 hrs. This mixture was run on the same column under similar conditions as above but A = 0.1 % aq HFBA and B = 0.08 % HFBA and B = 0.08 % HFBA in CH_3CN.

The peaks representing cleavage products were pooled, freeze dried, hydrolyzed and analyzed for amino acid composition.

B.10. Determination of Scissile Bonds for Collagenase

The procedure involved activating recombinant proMMP-1 (53 μg/mL) with 2 mM APMA at 37°C for 45 min. 20 μL of the activated enzyme was then added to 50 μL (3 μg/μL) of Nα_1-Ctelo and α_1-Ctelo-2. The pH was adjusted to 7.5 with Trizma. Controls for each substrate were done without any enzyme. The total volume of each was made up to 300 μL with buffer 1. Incubation was at 22°C for 72 hrs. Later on the experiments were repeated at 37°C for 49 hrs. The control and the digest were applied on a 0.45cm x 25cm C_{18} reversed-phase (Vydac 201TP104). The flow rate was 1 mL/min. Elution was isocratic in A for 10 min, followed by a gradient of 0.5 % B /min. For Nα_1C-telo A = 0.1% TFA in water and B = 0.1 % TFA in acetonitrile. Fractions under all the peaks were pooled, dried and analyzed.

B.11. Ac-PQPPQE-COOH as Competitive Product Analog of Collagen

[^{3}H] Acid soluble collagen 20 μL (8.63 μg) was equilibrated with Ac-PQPPQE-COOH. To each was then added 50 μL of telopeptidase 2. The total volume was made upto 240 μL with buffer 1. The final concentration of Ac-PQPPQE-COOH in the assay was 1.3 and 148 nM. No enzyme and enzyme controls were similarly prepared. The incubation was done at 27°C for 24 h. The rest of the procedure was as described in the telopeptidase assay (see section B.3.7.)

B.12. Design of Assay using synthetic peptide for Telopeptidase 2 / MMP3 activity

The assay curently used to detect telopeptidase activity uses tritiated collagen molecule as the substrate. This assay although very specific for detecting C-telopeptide cleaving activities is tedious, time-consuming and expensive. The concentration of substrate in the assay is very low, hence it is difficult to work at a concentration that is close to the K_m ($\simeq$ 25 μM, data not shown) which is essential in order to do kinetic study. Thus knowing the scissile bond a seven residue peptide Z-PQEKAHDNH$_2$ was made. Some of this peptide was cleaved by HF according to the procedure described previously. The product was then purified on large Synchropak C_{18} at a flow of 3mL/min. buffer A=0.1% aq. TFA, B=0.1% TFA in acetonitrile. Isocratic A was used for 10 min. and then 1% B /min was applied. The peptide eluted at 24% B.

To 72 μg of the peptide and 2.4 mg of the peptide still on the resin, in 60 μL of 1 X conc tris. was added 40 μL of MMP3 (114μg/mL, i.e. 0.196 units in 10μL). The digest was incubated in a water bath at 20°C. A 20 μL aliqout of the enzyme was added to each digest after 24 hrs and incubated for another 25 hrs.

The digest with the peptide on the resin was centrifuged and the supernatant loaded on RP-HPLC. Similarly the soluble peptide digest was also run on RP-HPLC. Any peak was hydrolyzed in 6 M HCl and analyzed.

The peptide was coupled to activated CH-Sepharose 4B according to the recommended Pharmacia procedure. The freeze-dried resin (200 mg) was swollen

and washed with 100 mL of 1 mM HCl on a sintered glass funnel. 10 mg of the peptide was dissolved in 100 μL of 0.01 M sodium bicarbonate, pH 8.0, and mixed with the resin for 90 min at 23°C. The coupled gel was then washed with 0.10 M Tris and 0.05 M NaCl, pH 8.0, alternating with 0.10 M sodium acetate and 0.50 M NaCl, pH 4.0, and stored at 4°C in 0.05 M Tris, 0.50 M NaCl, and 0.01 M $CaCl_2$, pH 7.5. A 50 μL aliquot was hydrolyzed in 6 N HCl and contained 2.29 μM of peptide per mL of resin.

40 μL of the peptide resin was incubated with 25 μL of MMP3 (660 μg/ mL, 3.6 units of telopeptidase) at 37°C for 3 hrs, centrifuged and the supernatant ran on RP-HPLC (analytical 25 cm X 10 mm Vydac C_{18} A = 0.1 % Aq HFBA, B = 0.1% HFBA in CH_3CN, gradient was 0.5%B/min & flow of 1mL/min).

B.13. Inhibition Studies

B.13.1. Human Plasma Alpha-Macroglobulin as Inhibitor of Telopeptidase

Human Plasma α_2 M (Sigma Lot No. 77F-9383 / No M-7151 / 90% purity) was used. The protein content when tested by biuret assay was found to be approximately 20%. This was tested for inhibition against telopeptidase activity in gingival explant medium using the telopeptidase assay described in the methods. Incubation was for 24.5 hrs.

B.13.2. Human Plasma Alpha$_2$-Macroglobulin as Inhibitor of Collagenase

25 μg of ASC and 3 μL of collagenase (fractions 12 to 14 off

chromatofocusing without dilution) and 4.47 pmoles of alpha$_2$-macroglobulin were incubated at 22°C for 22 1/2 hrs. in a total volume of 60 μL. 5% gels were run as described in the methods.

B.13.3. Effect of Synthetic Inhibitors on Matrix Metalloproteinases

Metalloproteinases play a major role in the pathogenesis of human disease, synthetic inhibitors should be of value in studying these processes and as potential therapeutic agents. Although they differ considerably in their protein substrate specificities the matrix metalloproteinase all have an essential zinc at their active site making them susceptible to inhibition by metal chelators. Some of the collagenase inhibitors are reviewed by Johnson, et al. 1987. One of the approaches to this goal is the synthesis of peptide analogues of the substrate cleavage site in which a metal binding functionality replaces the scissile carbonyl of the substrate. This strategy has yielded specific , high-affinity inhibitors of several zinc-dependent metalloproteinases, including angiotensin converting enzyme (Cushman, et al 1977), enkephalinase (Fournie-Zaluski et al. 1984) and thermolysin (Nishino et al. 1979).

To extend this approach to MMPs a series of di-, tri- and tetra peptide hydroxamates were synthesized (as described in methods section B.5.9.b) and tested extensively against MMP-2. Some of these inhibitors were also tested against MMP-1 and MMP-3. Based on these inhibitors, general conclusions can be drawn regarding the subsites of these enzymes.

Inhibition curves were prepared over a wide range of inhibitor concentrations and the results fitted to a sigmoid curve using a computer program (GraphPAD InPlot™, GraphPAD Software, San Diego, CA92121, USA) to derive IC_{50} values. K_i values were determined from Dixon plots (Dixon, 1953) at 4 substrate concentrations. A K_m for MMP-2 was determined by fitting the velocities measured over 1 hour at various values of [S], to a hyperbolic curve. The substitution of discrete measurements of product release after 1 hour for true initial velocity measurements was justified by the observation that the rate of product release was effectively linear for at least 1.5 hours.

B.14. Effect of Inhibitors on the Digestion of Collagen Fibrils by Fibroblasts

The collagen that was labelled with fluorescein isothiocyanate, coated and dried on tissue culture plates (see methods section B.1.5.) was used. To check that the collagen coated onto the plates retained its native structure, a comparison was made of its susceptibility to digestion with trypsin and bacterial collagenase. Trypsin (TRTPK; Worthington) and collagenase from Clostridium histolyticum (Advanced Biofactures Corporation) were dissolved at 0.2mg/mL and 4000 units/mL respectively in 0.025M Tris/0.01M calcium acetate, pH 7.4. This buffer (2mL), collagenase solution (0.1 mL of stock solution + 1.9 mL of buffer) or trypsin solution (25 μL + 1.975 mL of buffer) were added to duplicate wells on one plate which was then incubated at 37°C. Samples of the supernatant solution were removed at intervals of 1,2,4,6 and 24 hours and the fluorescence measured. After

24 hours the solution in the wells which had been incubated with trypsin was replaced by bacterial collagenase solution and incubation continued for a further 24 hours. Samples were again removed for measurement of solubilized fluorescence.

In order to establish that the collagen had been labelled covalently with FITC (which should react with some of the lysine and hydroxylysine residues), the peptides released by bacterial collagenase were fractionated by reversed-phase HPLC. Amino acid composition of three of the peptides which showed measurable fluorescence were determined, as described.

In preparation for addition of cells, 2.5 mL of phosphate-buffered saline (PBS) was added to each well and the plates incubated at 37°C for several days with daily changes of buffer, until the rate of release of fluorescence had declined to the background level (i.e. that of DMEM-10 alone). Each well was then seeded with $5x10^5$ cells in 1 mL of DMEM-10 and put into the incubator overnignt. This cell density was sufficient to produce confluence in about 24 hours, at which time the medium was normally replaced with DMEM without serum (DMEM-0). Medium containing the peptide hydroxamate inhibitor was added. Released fluorescence (excitation wavelength 490 nm, and emission 520 nm) was measured in the conditioned medium samples after brief centrifugation (Beckman Microfuge). The instrument (Perkin-Elmer LS-5 luminescence spectrophotometer) was zeroed using medium recovered from wells incubated without added cells. Hydroxyproline was measured in conditioned medium by gas-chromatography/mass spectrometry after

hydrolysis in 6N HCl at 110°C for 24 hours (Tredget et al., 1990).

When cell counts were required, fibroblasts were released from the plates by brief trypsinization and counted in a hemocytometer. Total cell counts were obtained under illumination with visible light and fluorescent cells were counted under uv illumination in a Zeiss epifluorescence microscope.

III RESULTS

A. Purification of Human Gingival Fibroblast Telopeptidase

Activation of concentrated medium with trypsin-agarose resulted in considerable increases in telopeptidase, collagenase and gelatinase activity (6-, 3-, and 22-fold respectively) , indicating that all three enzymes are present primarily in latent forms. The results of the purification for telopeptidase are sumarized in Table 1.

A.1. Gel Filtration Chromatography

The elution profile for concentrated, activated medium on agarose is shown in Fig 9. Telopeptidase activity eluted from agarose A-0.5m with an apparent M_r of approximately 69 000. The major peak of gelatinase activity eluted with an apparent M_r of 160 000, Collagenase eluted at an apparent M_r of 35 000. The elution patterns of the above three activities did not follow the patterns for digestion of either of the nonspecific proteinase substrates azocoll or azocasein, indicating the importance of testing for the activity of the protein of interest. The three MMPs mentioned above were not very well resolved from one another. The fractions under the telopeptidase peak were pooled, concentrated and desalted for further purification.

Fig. 9. Gel chromatography of concentrated and activated fibroblast medium on agarose A-0.5m. Sixty millilitres was applied to a 5 x 99 cm column of A-0.5m (200-400 mesh). The effluent was monitored for protein at 280 nm. Fractions of 10 mL were collected at a flow rate of 33 mL/hr. A: shows the elution profile of telopeptidase (×—×), gelatinase (•—•) and collagenase (▲–▲) activity. B: shows the elution profile of azocasein (⊙—⊙) and azocoll (■—■) digesting activities. Horizontal bar denotes fractions pooled for further purification. Arrows indicate elution positions and MWs of protein standards: aldolase (158 000), bovine serum albumin (66 000), chymotrypsinogen A (25 000), and ribonuclease (13 700).

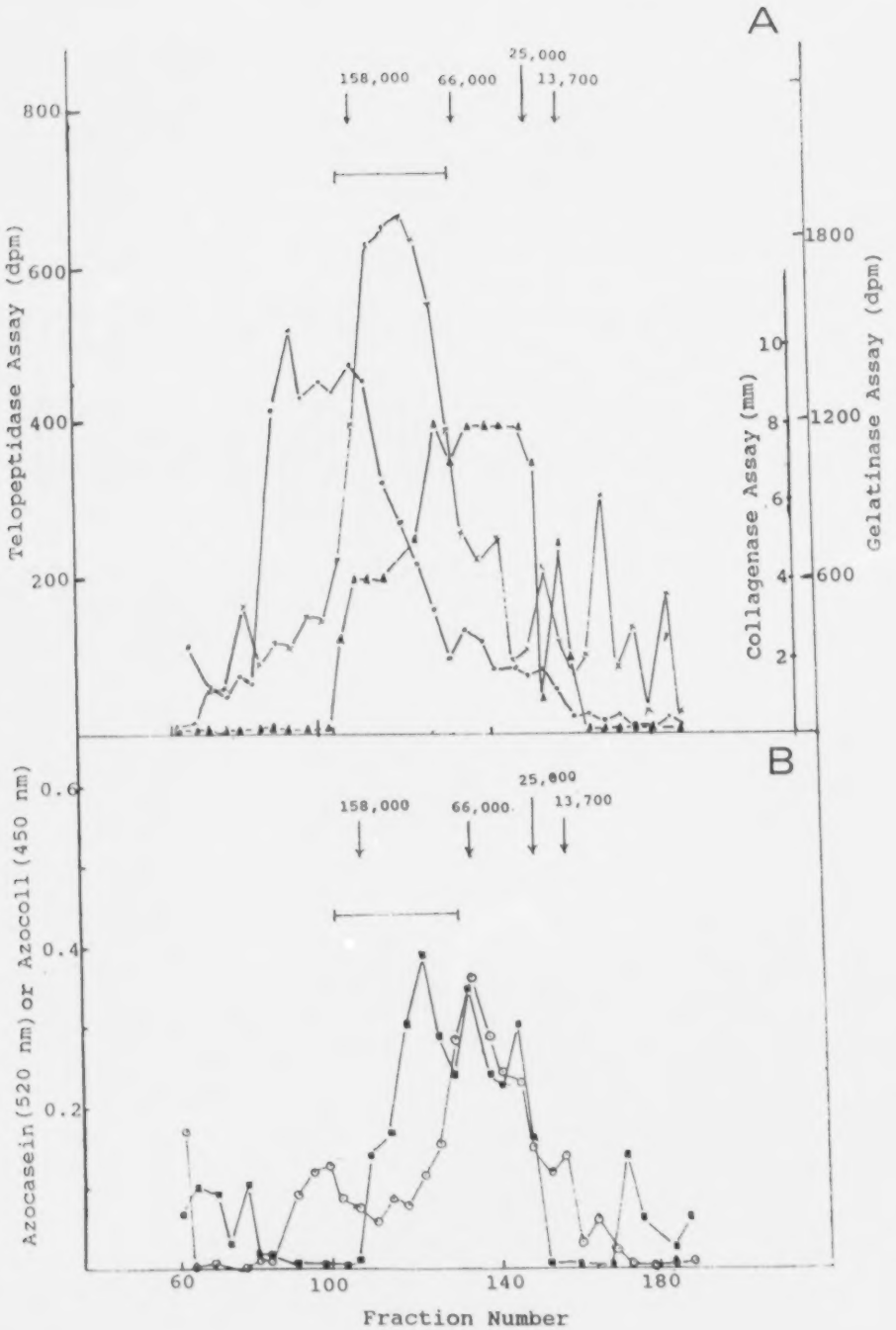

A
158,000
66,000
25,000
13,700
800
600
400
200
Telopeptidase Assay (dpm)
1800
1200
600
Gelatinase Assay (dpm)
10
8
6
4
2
Collagenase Assay (mm)
B
158,000
66,000
25,000
13,700
0.6
0.4
0.2
Azocasein (520 nm) or Azocoll (450 nm)
60
100
140
180
Fraction Number

Table 1

1. Purification of Telopeptidase from Gingival Fibroblast Culture Medium[a]

Step	Protein (mg)	Activity (units)	Recovery (%)	Specific Activity (U/mg)	Purificatio Factor (nfold)
Concn. medium 2L	2000	9985	100	4.99	1
A 0.5	59	3914	39.2	66.4	13.3
Chromatofocusing	20.9	1953	19.6	93.5	18.7
Fraction 2[b]	1.0	192	1.92	192	39
Fraction 3[b]	1.1	281	2.81	256	51.2
Telopeptidase 1[c]	0.062	32	0.32	516	103.4
Telopeptidase 2[c]	0.13	35.6	0.36	273.9	54.0

a - see scheme 3
b - fractions off chromatofocusing
c - Purified fractions after second hydrophobic interaction chromatography

A.2. Chromatofocusing

The elution profile of chromatofocusing is shown in Fig 10. On chromatofocusing telopeptidase activity was found to be present in two main peaks, one at pH of 5.8 (Fractions 36-58) and the other at a pH of 4.3 (Fractions 72-87). Gelatinase activity was present in several peaks, some of which were not resolved from those of telopeptidase or collagenase. Each fraction was neutralized within half an hour of elution using Trizma because the enzymes were found to rapidly inactivate at low pH. Moreover they slowly lose activity in Polybuffer, (only 12% of gelatinase activity remains after 7 weeks at 4°C). Collagenase which has a high isoelectric point (pI of 7.4) elutes straight through the column. Gelatinase, as well as some telopeptidase activity is present in these fractions (Fractions 12 to 14).

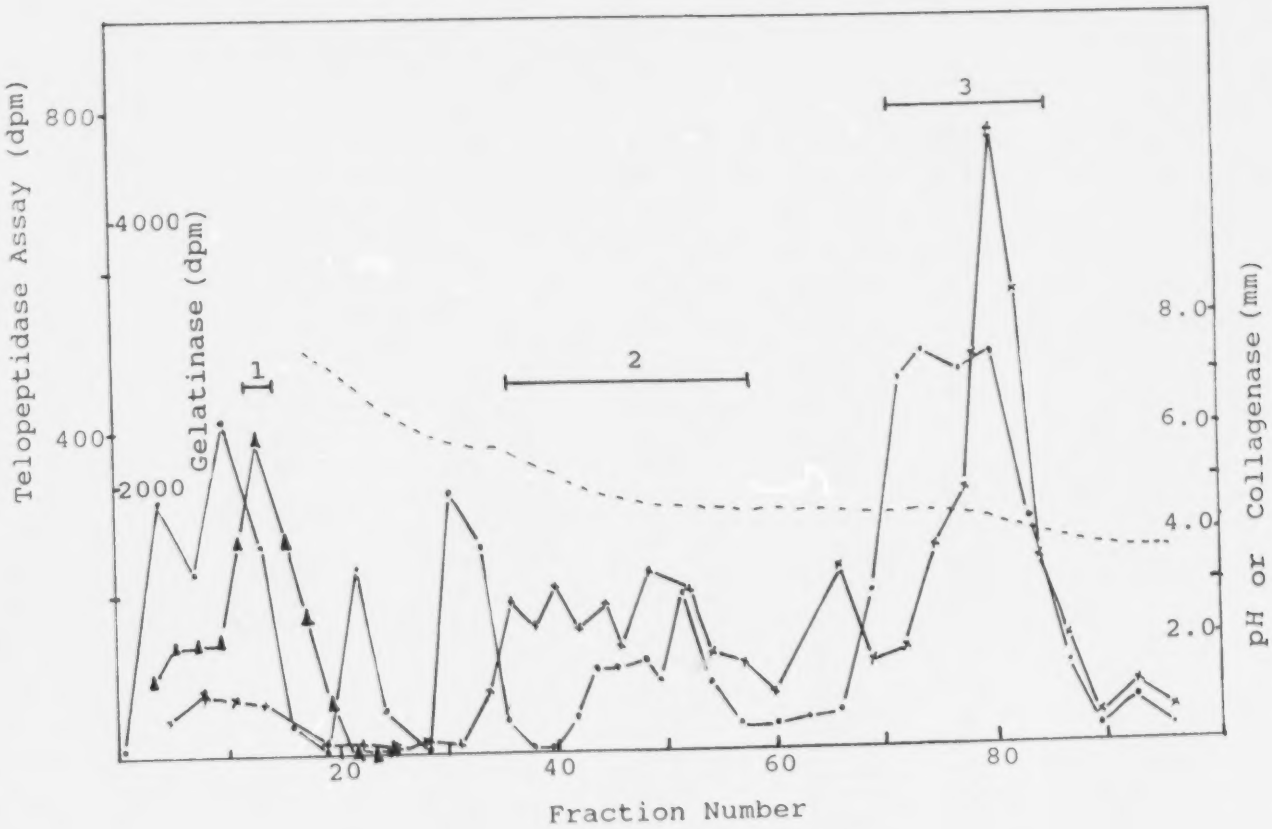


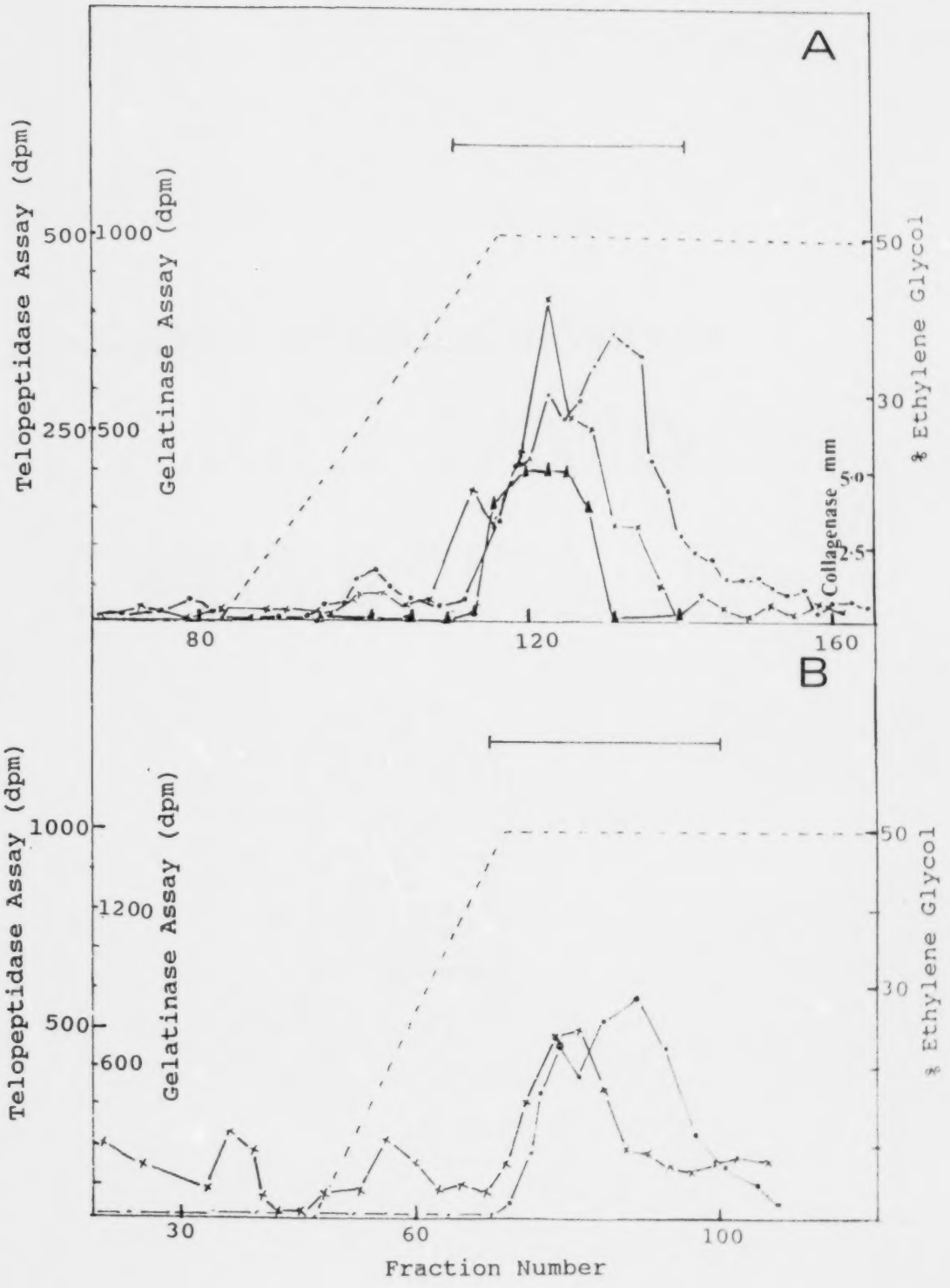
Figure 10. Chromatofocusing of telopeptidase-containing fractions from A-0.5m (see Fig 9). 52 mL was applied to a 1 X 17 cm column of PBE 94 equilibrated with 0.025 imidazole /0.05% Brij 35 (pH 7.4) and eluted with 340 mL of polybuffer 74 (pH 3.9) at a flow rate of 35 mL/h. Fractions (4.5 mL) were collected and assayed for telopeptidase (x—x 20 μL/assay at 21°C for 17.5 hrs), gelatinase (•—• ; 5 μL/assay at 37°C for 1 h), collagenase (▲—▲ ; 5μL/assay of 10 x diluted fraction at 37°C) and pH (---). Horizontal bars denote fractions pooled for further study.

Since collagenase is known to have gelatinase activity, this activity could be that of collagenase itself (Welgus et al., 1982). The telopeptidase activity in these collagenase-activity-containing fractions was very weak when tested immediately after elution but was greatly increased when tested after a few days (see discussion). Collagenase-activity-containing fractions 12 to 14 were pooled at this stage and used as purified collagenase (Fraction 1 off Chromatofocusing/see scheme 3 in methods section B.6). It was diluted 3.6 times with buffer 1 (see methods) when used for assay. Fraction 1 was stored at 4°C and was stable for at least 2 years. The two telopeptidase-containing peaks (Fraction 2 and Fraction 3) were pooled and treated separately from this point on. Due to instability of the enzymes in Polybuffer the samples were chromatographed on Phenyl-Sepharose as soon as possible (within two days).

A.3. Hydrophobic Interaction Chromatography

On application to Phenyl-Sepharose in ammonium sulphate (20% saturation), both gelatinase and telopeptidase bound tightly and eluted with 50% ethylene glycol (Fig 11). The two activities however did not resolve. In order to carry out the telopeptidase assay it was necessary to remove ammonium sulphate; hence 40 μL aliquots of 4-5 fractions were pooled and applied to a 1 x 4 cm (2 mL) Sephadex G-25 column eluted with buffer 1 (see methods). Although fraction 2 did not show noticible collagenase activity after chromatofocusing, there was significant collagenase activity after this Phenyl-Sepharose chromatography step (Fig 11A).

Figure 11. Phenyl-Sepharose chromatography. A: chromatography of telopeptidase containing fractions from fraction 2 off chromatofocusing. B: chromatogram of telopeptidase containing fractions from fraction 3 off chromotofocusing. Fractions (1.5 mL) were collected at a flow rate of approximately 13 mL/hr. and assayed for telopeptidase (×—× ; 50 μL/assay at 21°C for 24 hrs), collagenase (▲—▲ ; 5μL/assay at 37°C for 24 hrs), and gelatinase (•—• ; 10 μL/assay at 37° for 1 h) activity. For telopeptidase assay, aliquots from 4 to 5 tubes were combined and applied to a 1 x 4 cm Sephadex G-25 column eluted with buffer 1, to remove ammonium sulphate which interfered with the assay. Horizontal bars denote fractions pooled for further studies. Fraction 2 (panel B) did not show any collagenase activity.


A
B
Telopeptidase Assay (dpm)
Gelatinase Assay (dpm)
% Ethylene Glycol
Collagenase mm
500
1000
250
500
50
30
5.0
2.5
80
120
160
1000
1200
500
600
30
60
100
Fraction Number

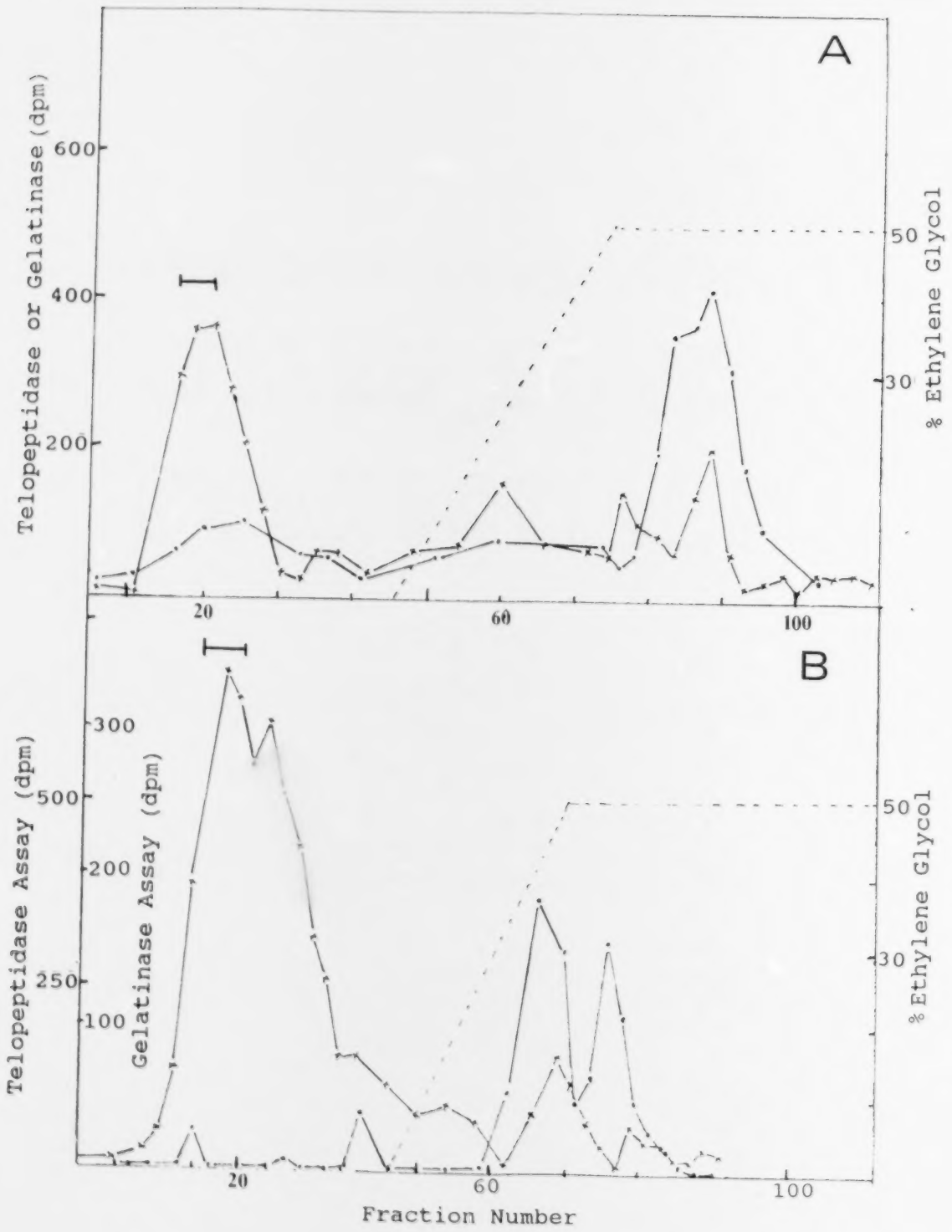
Fraction 3 off chromatofocusing after Phenyl Sepharose did not show the presence of collagenase (Fig 11B). Fractions denoted by bars were pooled and rechromatographed on Phenyl-Sepharose without ammonium sulphate.

Rechromatography of the individual peaks on Phenyl-Sepharose without ammonium sulphate was done in an attempt to resolve telopeptidase from gelatinase (Fig 12). Without ammonium sulphate most telopeptidase activity did not bind to the column whereas gelatinase bound tightly and required 50% ethylene glycol for elution. Fraction A (see scheme 3) when rechromatographed on Phenyl-Sepharose without ammonium sulphate (Fig 12A) showed several peaks with gelatinase activity. The telopeptidase containing fractions also showed weak gelatinase activity. These fractions 17-20 were pooled for further study and referred to as telopeptidase 1. On rechromatography of Fraction B (see scheme 3 and Fig 12B), the telopeptidase containing fractions were relatively free of gelatinase activity. These fractions 15-20 were pooled further studies and referred to as telopeptidase 2. Both fractions were stored at 4°C.

A.4. Optimum pH for Proteodermatan Sulphate digesting activity of Telopeptidase 2

Figure 13 shows a plot of percent digestion of proteodermatan sulphate against pH. The pH optimum was around 7. The pH measurements were not done below pH 4.85 or above 9.0 because the substrate has low solubility at pH extremes.

Figure 12. Rechromatography of telopeptidase-containing fractions on Phenyl-Sepharose, without added ammonium sulphate. A: chromatography of fractions from fraction 2 off chromatofocusing after Phenyl-Sepharose. B: chromatography of fractions from fraction 3 off chromatofocusing after Phenyl-Sepharose. Conditions were as in Fig 11. except that no ammonium sulphate was added instead sample and column were equlibrated in buffer 1 and elution was performed with a gradient formed from 15 mL of buffer 1 and 15 mL of 50% ethylene glycol in buffer 1 (see methods). Telopeptidase activity (×—×; 50μL/assay at 22°C for 24 hrs), and gelatinase activity (•—•; 10μL/assay at 37°C for 1 h). The bars denote pooled fractions.


A
Telopeptidase or Gelatinase (dpm)
600
400
200
50
30
% Ethylene Glycol
20
60
100
B
Telopeptidase Assay (dpm)
500
250
Gelatinase Assay (dpm)
300
200
100
50
30
% Ethylene Glycol
20
60
100
Fraction Number

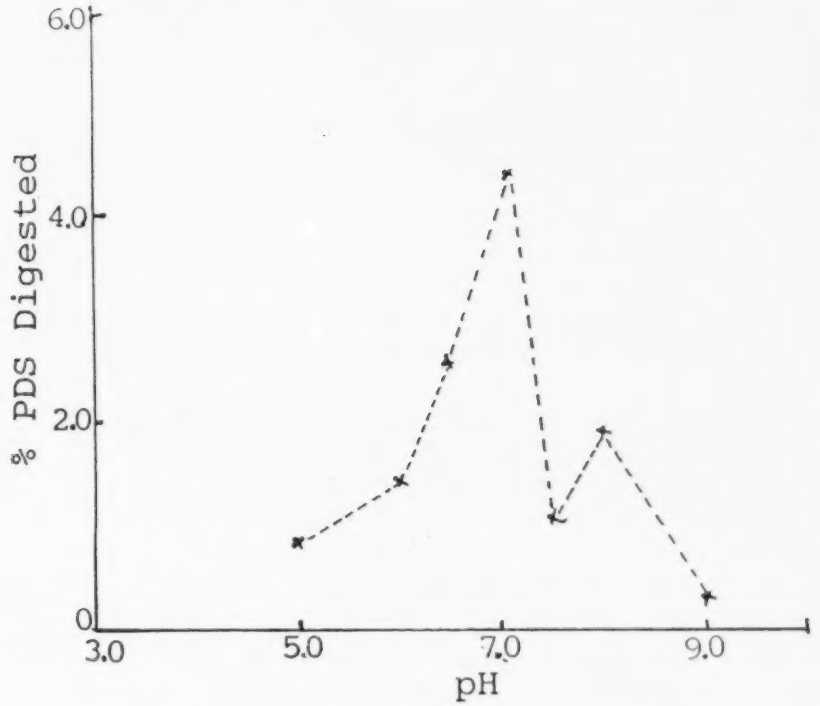


Fig 13. Effect of pH on proteodermatan sulphate-degrading activity of telopeptidase-2 .

B. Large Scale Incubations with Acid Soluble Collagen

B.1. Large Scale Incubation of Collagen with Telopeptidase 1 and 2

B.1.1. 5% Polyacrylamide Gels

The gels of collagen digested with telopeptidase 1 showed the presence of collagenase-activity as seen by the release of $\alpha_{3/4}$ and $\alpha_{1/4}$ fragments from collagen (Fig 14), whereas those digested with Telopeptidase 2 were free of any collagenase activity.

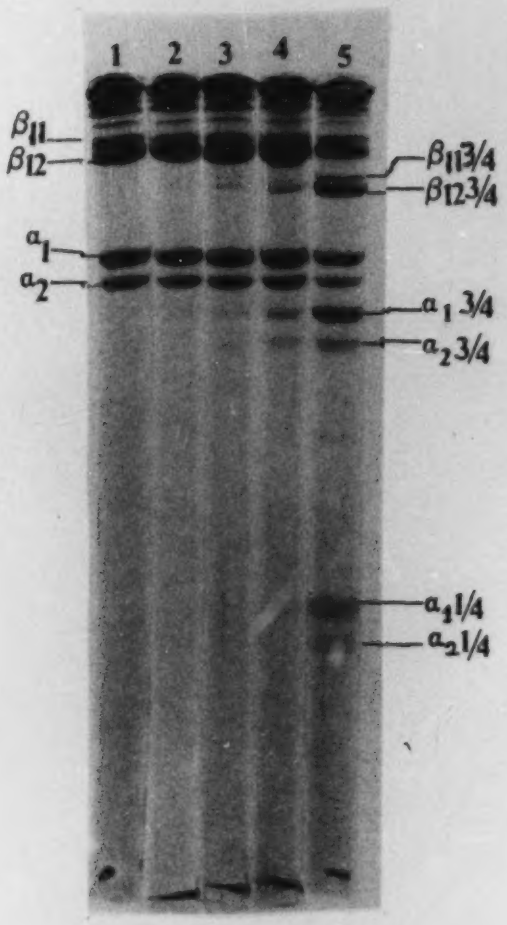


Figure 14. 5% Polyacrylamide gel electrophoresis of acid soluble collagen. Gel #1: control ASC; Gel #2: ASC digested with telopeptidase 2 and gels 3 to 5 collagen digested with different fractions of telopeptidase 1.

B.1.2. Cyanogen Bromide Digest

The CNBr peptides of the collagen (see Fig 1A) incubated with telopeptidase 1 and 2 showed characteristic changes in the α_1-CB6 region (Fig. 15 & 16). Gels of collagen digested with both samples showed a decrease in the α_1CB6a band intensity corresponding to the entire C-telopeptide. Gels of collagen digested with telopeptidase 1 showed a corresponding increase in the α_1-CB6c band in comparison to the control (indicating that most of the C-telopeptide was lost). The peak preceding α_1CB6a is that of α_1CB7' which is due to some collagenase present in the sample as seen in the 5% polyacrylamide gels (Fig 14). The gels digested with telopeptidase 2 showed an increase in the α_1CB6b indicating that part of the C-telopeptide had been lost. Figure 16 shows the scans of the 12 % gels where the absorbance is plotted against the relative mobility. Since the tritium label is incorporated by reduction of the lysine-derived aldehyde that is residue 17 in the telopeptide (Scott & Goldberg 1983), this suggests that the cleavage must have occurred on the N-terminal side of this residue in the C-telopeptide. Thus from this experiment one can conclude that telopeptidase 1 cleaves closer to the helix, while the cleavage by telopeptidase 2 would be N-terminal of, but closer to the Lys_{17} in the C-telopeptide.

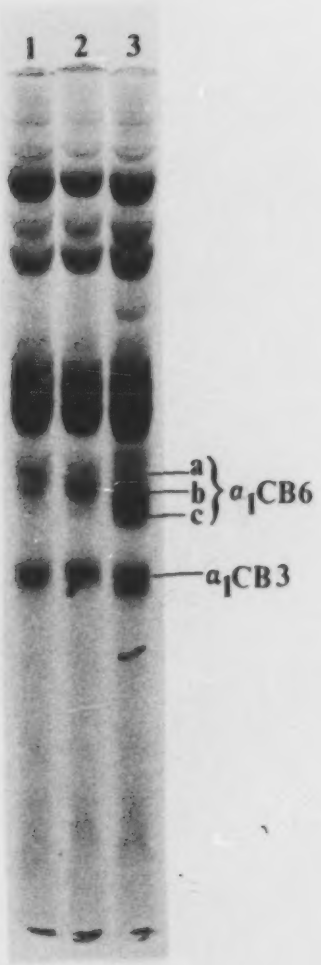


Figure 15. Photograph of the 12 % polyacrylamide gels of the CNBr peptides obtained from tritiated collagen. Gel #1 control, Gel #2 Collagen digested with telopeptidase 2 and Gel #3 collagen digested with telopeptidase 1. The dye used was bromophenol blue. a, b & c denote the forms of the telopeptide, which is intact in α_1CB6a; partially removed in α_1CB6b; and almost completely removed in α_1CB6c.

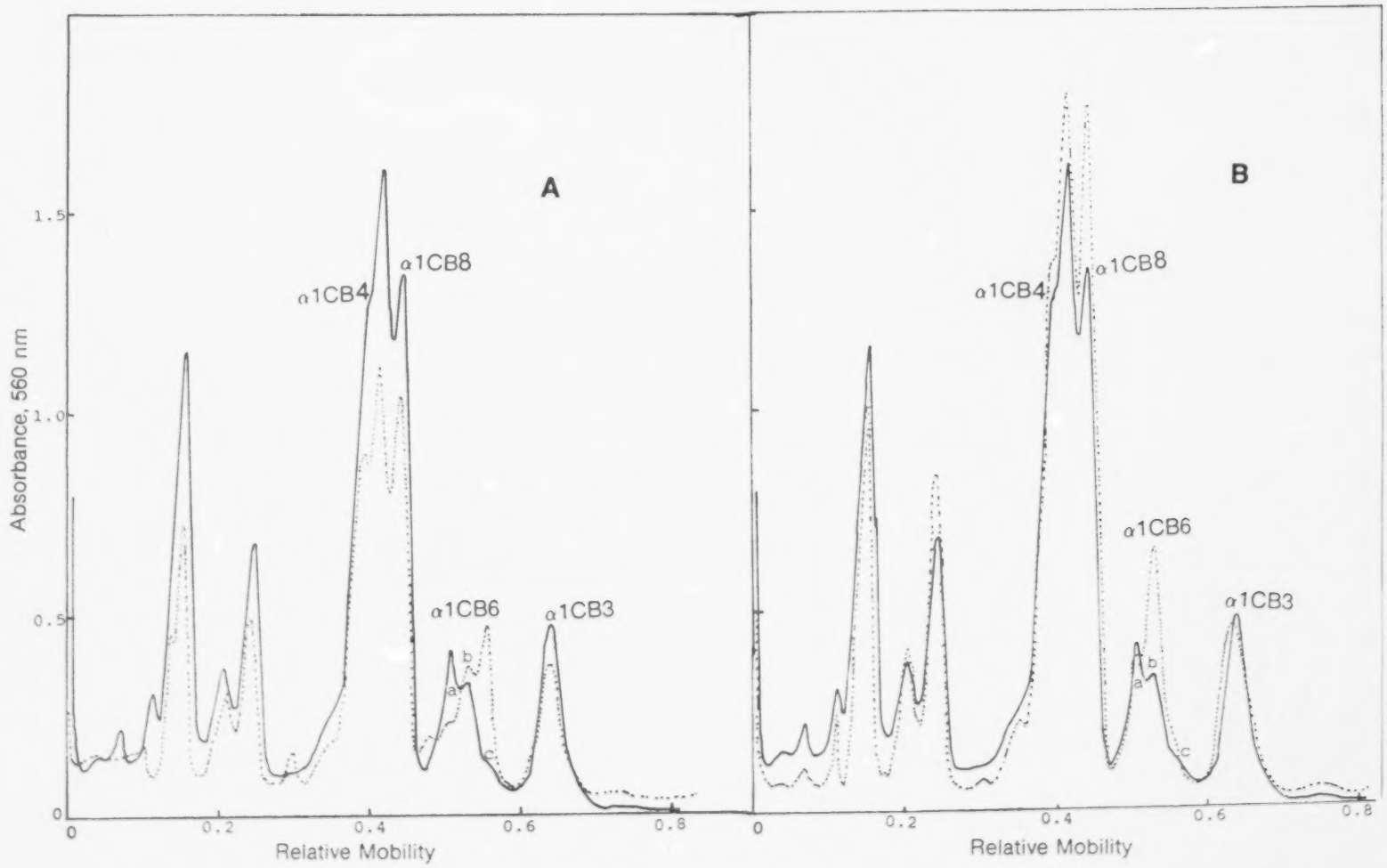


Figure 16. Gel electrophoresis of CNBr peptides obtained from soluble tritiated collagen. Panel A: the no-enzyme control (———)and digest of telopeptidase 1 (-----); panel B: no enzyme control (———), digest of telopeptidase 2 (-----). For details see legend to Fig 15.

B.2. Large Scale Incubation of Collagen with Purified and Recombinant Stromelysin (MMP-3)

This experiment was done with purified and recombinant MMP-3 in order to test if the collagen C-telopeptide is cleaved by purified and recombinant MMP-3 and to compare the effects of these enzymes with that of telopeptidase. The CNBr peptides of the collagen incubated with these enzymes showed different patterns in the α_1CB6 region. Figure 17 shows a comparison of the CNBr data for the three enzymes. Gels of collagen digested with all three enzymes showed a decrease in the α_1CB6a band intensity corresponding to the entire C-telopeptide. Telopeptidase 2 digest shows an increase in the α_1CB6b band (Fig.17B). Purified MMP-3 digest showed an increase in the α_1CB6b as well as the α_1CB6c peak as compared to the control (Fig 17 C & D). The peak preceding α_1CB6a is that of α_1CB7' which is due to some collagenase present in the sample. The presence of collagenase in this purified MMP-3 preparation was also confirmed by the release of $\alpha_{1/4}$ and $\alpha_{3/4}$ fragments from collagen. Recombinant MMP-3 also showed an increase in the α_1CB6b and α_1CB6c peaks (Fig 17F). In the telopeptidase 2 digest (Fig 17B) if there is an increase in the α_1-CB6c band, it is insignificant.

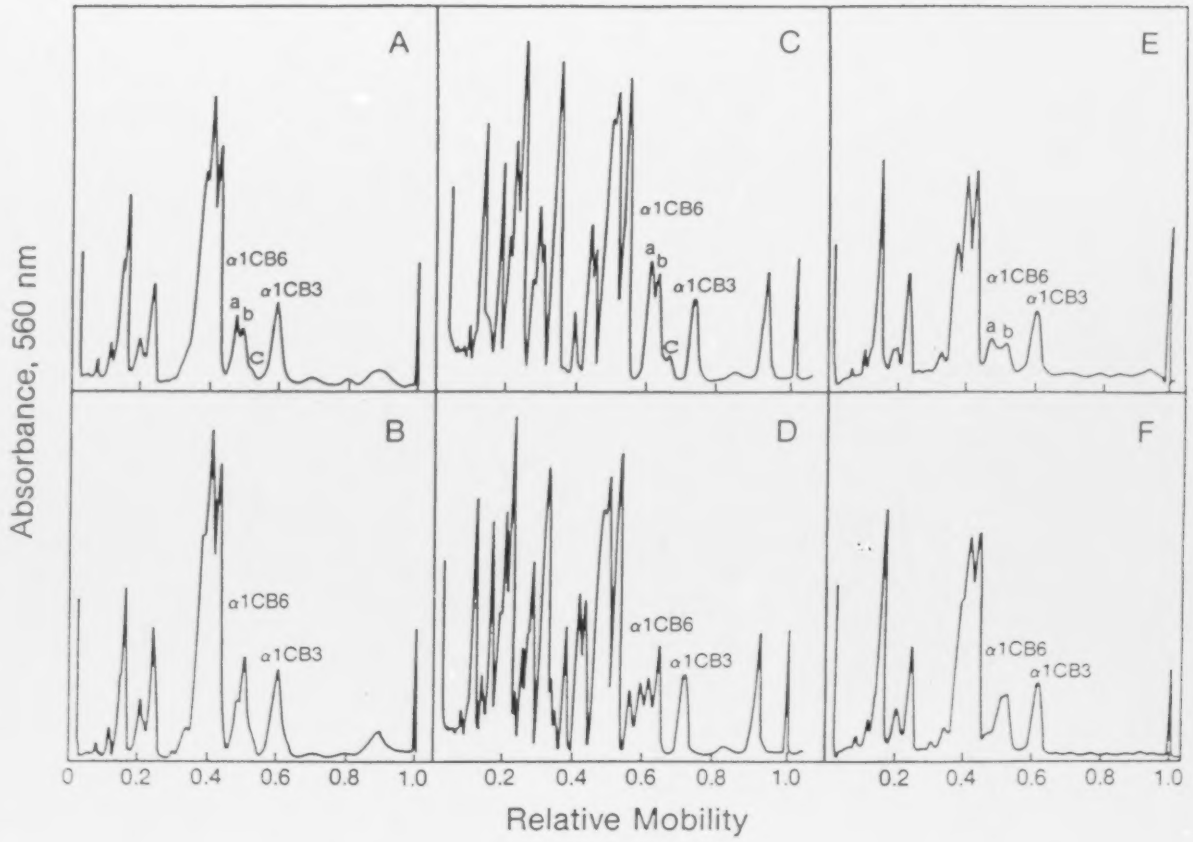


Figure 17. Gel Electrophoresis on 12% polyacrylamide SDS gels of CNBr peptides of acid soluble type I collagen digested with B:telopeptidase 2, D: purified MMP3 (Nagase) F: recombinant MMP3. A,C & E are no enzyme CNBr-digested controls (acid soluble collagen known to contain intact C-terminal telopeptide) for B, D & F respectively. These three experiments were run and analyzed at different times, hence the differences in appearance of the controls.

C. Determination of the Scissile Bonds of Telopeptidase

C.1. Action of Explant Culture Medium on the Entire Synthetic C-telopeptide

The pattern on running this digest on RP-HPLC was very complex (Fig.18). In the digest, the absorbance of the major peak decreased considerably as compared to the control which indicates that digestion of the peptide had occurred. On analysis none of the peaks could be clearly assigned to fragments of the substrate. After several attempts (of what seemed to be a meaningless experiment) it was concluded that the result could be attributed to the fact that the culture medium contains a mixture of several peptide digesting activities, hence the experiment was repeated with purified telopeptidase 2.

C.2. Action of Purified Telopeptidase 2 on the Entire C-telopeptide

The RP-HPLC profile of this digest was again complex (Fig 19) and not all of the peaks analyzed could be assigned to peptide fragments of the substrate. Peak 1 eluted at 14 % B and contained the peptide Ac-GPPSGGYD-, peak 2 eluted at 14.5 % B and showed on analysis that it could be assigned to the peptide -KAHDGGRYY-NH_2, while peak 5 eluted at 30 % B and appeared to contain a mixture of peptides, one being the entire substrate and the other that of the peptide Ac-GPPSGGYDLSFLPQPPQE- (Table 2). The results of the above experiment suggest that there are at least two cleavages (one between E and K and the other probably between D and L).

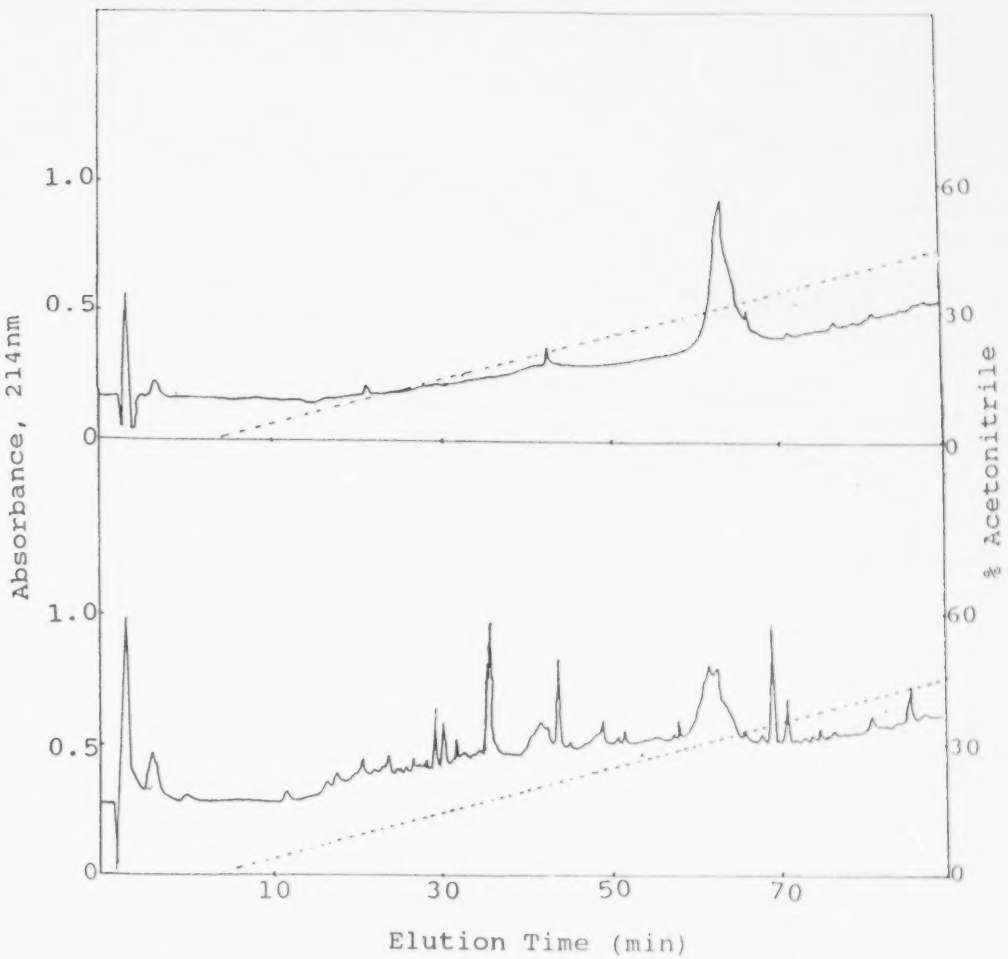


Figure 18. RP-HPLC of the entire C-telopeptide (α_1C-telo-1) digested with explant culture medium. Column used was 0.45cm x 25cm C_{18} reversed-phase column (Vydac 201TP104). Panel A: control peptide α_1C-telo-1, B: α_1C-telo-1 digested with activated conditioned medium. Eluents, A:0.1% aqueous TFA, B:0.1% TFA in acetonitrile. Flow rate = 1 mL/ min. Elution was isocratic in A for 10 min followed by a linear gradient of 0.83 % B/ min.

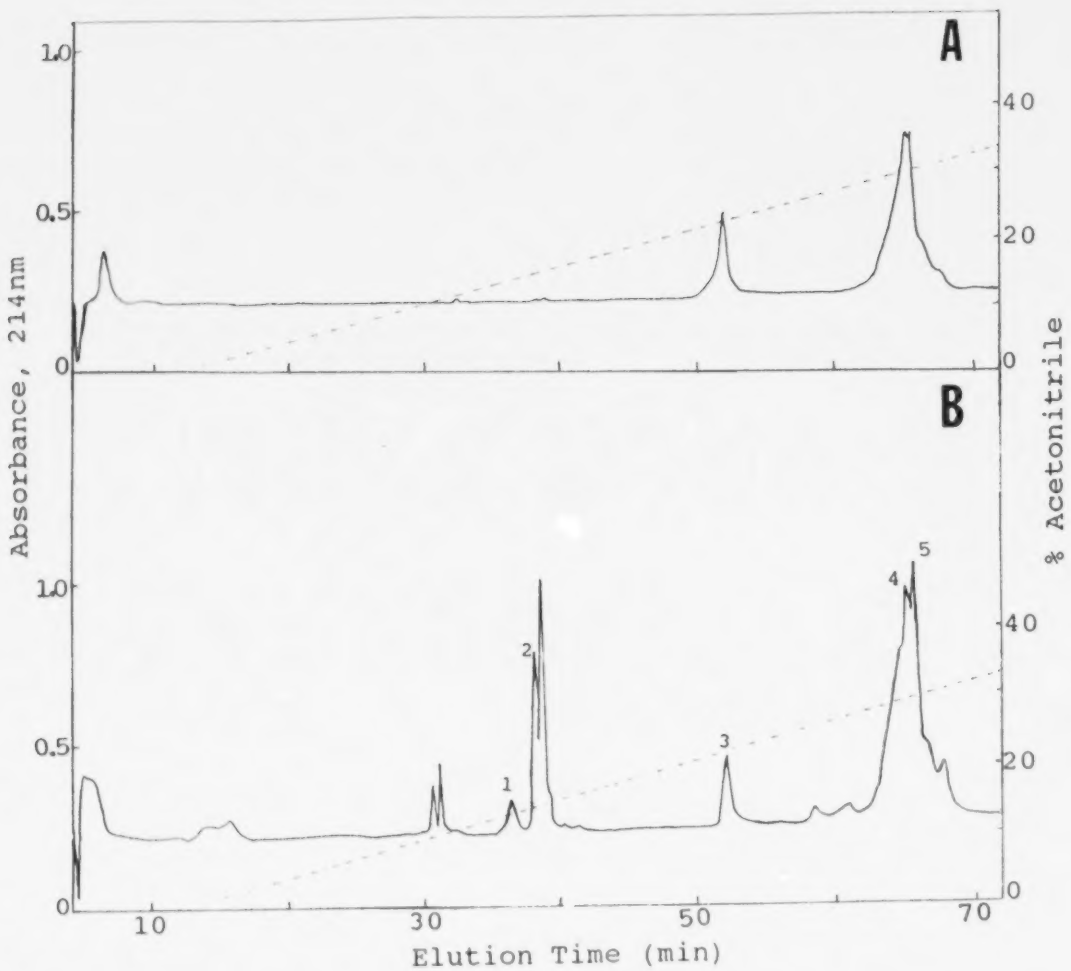


Figure 19. RP-HPLC of the entire synthetic α_1-Ctelo-1 peptide digested with purified telopeptidase 2. The column used was 0.45cm x 25cm C_{18} reversed-phase column (Vydac 201TP104). Panel A: control peptide, panel B:digest. Eluents A = 0.1% aq TFA & B: 0.1% TFA in CH_3CN. Flow rate was 1 mL/min and a gradient of 0.5% B/ min was applied after eluting isocratically with A for 10 min. Absorbance was monitored continously at 214_{nm}. Fractions corresponding to each peak were pooled, dried and analyzed. Only the peaks found on analysis to contain peptide(s) are labelled.

The other fragments (-LSFLPQPPQE-, -LSFLPQPPQEKAHDGGRYY-NH_2, and Ac-GPPSGGYDLSFLPQPPQE-) predicted to be formed as a result of these cleavages could not be found. The fractions under peak 3 (Fig 19B) did not show the presence of any amino acids. This peak was found to be present in the control (Fig 19A) as well as the blank (gradient run without any sample) run (not shown). This observation points to the importance of washing the column thoroughly and doing a blank as well as a control run.

From this experiment it was concluded that the synthetic peptide (27 amino acids long) contains several deletion peptides (see Fig 19 & Table 2, Peak 4) which, along with the digestion products, gave a complex unresolvable pattern on RP-HPLC. If possible, the substrate should be further purified before use. The substrate used in this is long and multiple cleavages could lead to a complex pattern. Thus a smaller substrate needed to be synthesized.

Although the above experiment was not conclusive, it clearly indicated that the peptide substrate was cleaved by telopeptidase. Considering the amino acid analysis of fragments isolated by RP-HPLC (Table 2, peak 1 and 2), and knowing that the cleavage occurrs at the N-terminal of Lysine 17 (based on radioactive CNBr results) it seems that there were cleavages at the Glu-Lys and the Asp-Leu peptide bonds. Thus a peptide substrate including one of these sequences needed to be made. The sequence Ac-PQPPQEKAHDGGRYY-NH_2 seemed appropriate. The two tyrosines at the C-terminal would help detect the peptide or the cleavage product at 280_{nm}. The sequence PQPPQ is unique to the non-helical

C-telopeptide of type I collagen hence it was included in the synthetic substrate prepared.

The N-terminal part of the C-telopeptide (Ac-GPPSGGYDLSFL-NH_2) was synthesized to include the Asp-Leu peptide bond. The two peptides were synthesized separately so as to avoid complex patterns on HPLC during purification of the fragments produced by the action of the enzymes.

TABLE 2

2. Peptides produced by the action of telopeptidase 2 on α_1C-telo-1

HPLC peak #	Peptide	% B	Amino acid composition
Peak 1	Ac-GPPSGGYD-	14.0	G P P S G 1.14 1.01 1.01 1.14 1.14 G Y D 1.14 0.6 0.8
Peak 2	-KAHDGRYY-NH_2	14.5	K A H D G G 0.8 0.95 0.9 0.8 1.13 1.13 R Y Y 0.95 1.12 1.12
Peak 4	SGGYDLSFLPQPP QEKAHDGGRYY-NH_2	29.5	S G G Y D L S 1.14,1.1,1.1,0.8,0.9,1.12,1.14 F L P Q P P Q 1.02,1.12,1.0,0.95,1.0,1.0,0.95 E K A H D G G 0.95,1.15,0.9,1.0,0.9,1.1,1.1 R Y Y 1.0,0.8,0.8
Peak 5	AcGPPSGGYDLSF LPQPPQEKAHDG GRYY-NH_2 + AcGPPSGGYDLSF LPQPPQE-	30.5	G P P S G G Y D 2.2,2.03,2.03,2.28,2.2,2.2,1.6,1.6, L S F L P Q P P 1.8,2.28,2.0,1.8,2.03,1.9,2.03,2.03, Q E K A H D G G R 1.9,1.9,1.0,1.12,1.0,0.8,1.1,1.1,1.12, Y Y 0.8,0.8

C.3. Action of Purified Telopeptidase 2 on Ac-PQPPQEKAHDGGRYY-NH_2 (α_1C-telo-2)

C.3.1. Separation of Fragments on RP-HPLC using Trifluoroacetic acid as Ion-pairing Agent

On RP-HPLC the control showed a single peak at 17.5 % B (Fig 20), whereas the digest showed two distinct peaks, one at 13.5 % B and the other at 17.5 % B. On amino acid analysis (Table 3) both the peak at 13.5 % and that at 17.5 % gave the same ratio of amino acids as the entire peptide substrate. Since the intact substrate cannot run at two different places in the gradient, digestion must have occurred. The fragments produced by the enzyme might have similar hydrophobicity and hence might not have resolved on RP-HPLC under these conditions. Since the undigested peptide elutes at 17.5 %, the fragments must have eluted together at 13.5 % B.

Table 3

3. Peptides of α_1C-telo-2 by telopeptidase 2 on RP-HPLC using TFA

HPLC peak #	% B	Amino Acid Composition	nmoles of peptide
1	13.5	P Q P P Q E K A H 1.0,0.9,1.0,1.0,0.9,0.9,0.9,0.9,1.1, D G G R Y Y 0.9,1.0,1.0,1.1,1.1,1.1	6.072
2	17.5	P Q P P Q E K A H 1.0,0.9,1.0,1.0,0.9,0.9,1.0,1.1,1.1 D G G R Y Y 0.9,1.1,1.1,1.2,0.9,0.9	7.14

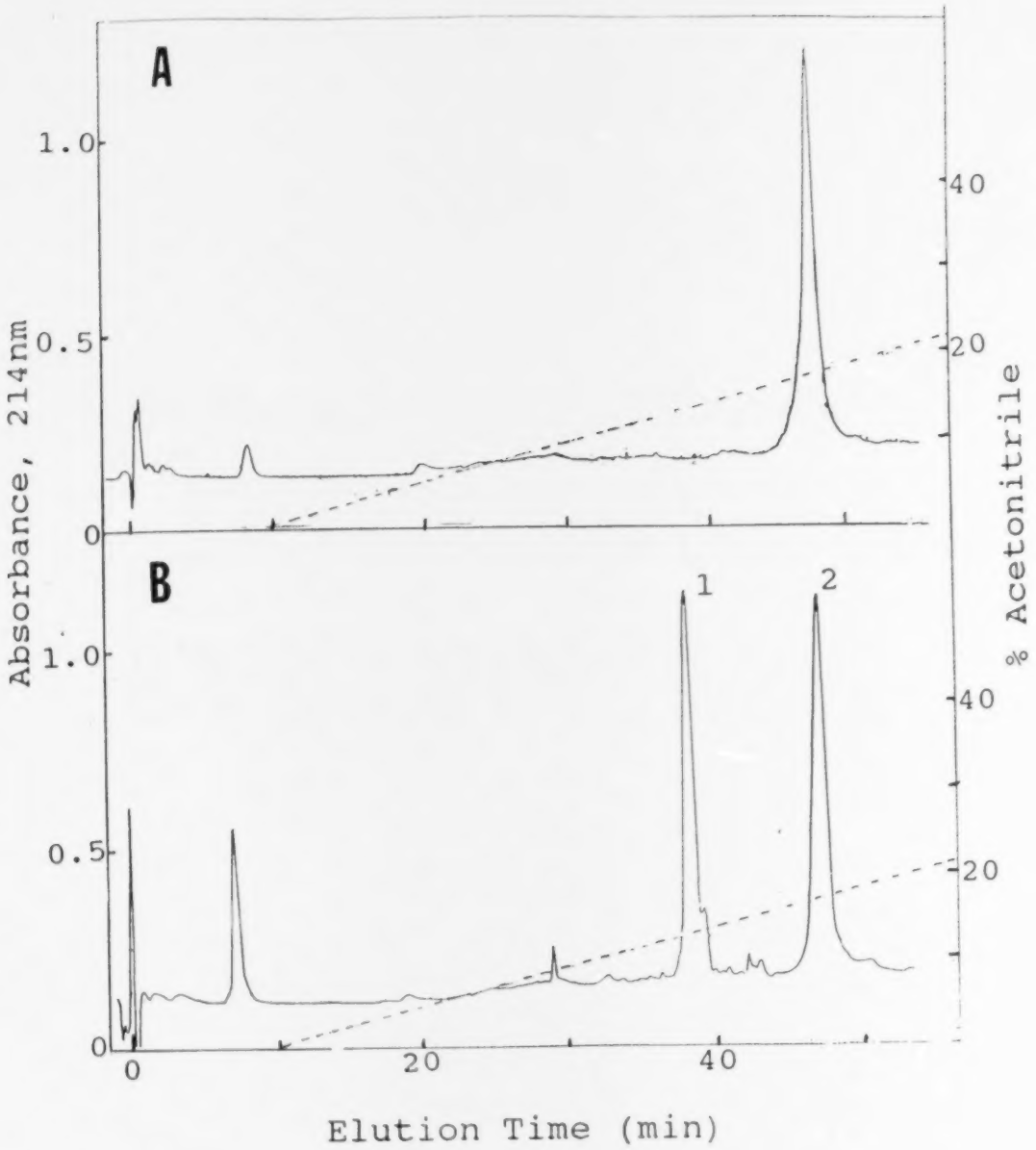


Figure 20. RP-HPLC of Ac-PQPPQEKAHDGGRYY-NH_2 (α_1C-telo-2) digested with purified telopeptidase 2 using TFA as ion-pairing agent. Panel A: control peptide, Panel B: 120 μL of the digest. Column and conditions as for Figure 19. Both the peaks (1 & 2) were found on analysis to contain the peptide Ac-PQPPQEKAHDGRYY-NH_2.

C.3.2. Separation of Fragments on Strong Cation Exchange HPLC

The sequence of the substrate is Ac-PQPPQEKAHDGRYY-NH_2. If as we think the enzyme cleaves between Glu and Lys (see results section C.2.), then the fragments produced would be

Ac-PQPPQE-COOH net charge at pH 6.5 = -2

$_2$HN-KAHDGGRYY-NH_2 net charge at pH 6.5 = +2.5

The substrate (α_1C-telo-2) does not have any net charge at pH 6.5. Thus these would be better separated by cation exchange HPLC.

When run on cation exchange HPLC the undigested peptide came straight through the column (Fig 21). In the run with the digest three peaks were obtained, at 0%, 4% B and 35% B. The fractions under these peaks, were collected, dried and rerun (for desalting) on RP-HPLC (Fig 22).

The breakthrough fractions (peak 1) was the undigested peptide and eluted at 17.5 %B on RP-HPLC (Fig 22A). The fractions under the peak that eluted at 4 % B (peak 2) on cation exchange could not be detected on RP-HPLC (not shown). The peak that eluted at 35 % B (peak 3) on the cation exchange column, when run on RP-HPLC (Fig 22B) eluted at 13.5 % B and on analysis proved to be H_2N-KAHDGGRYY-NH_2 (Table 4).

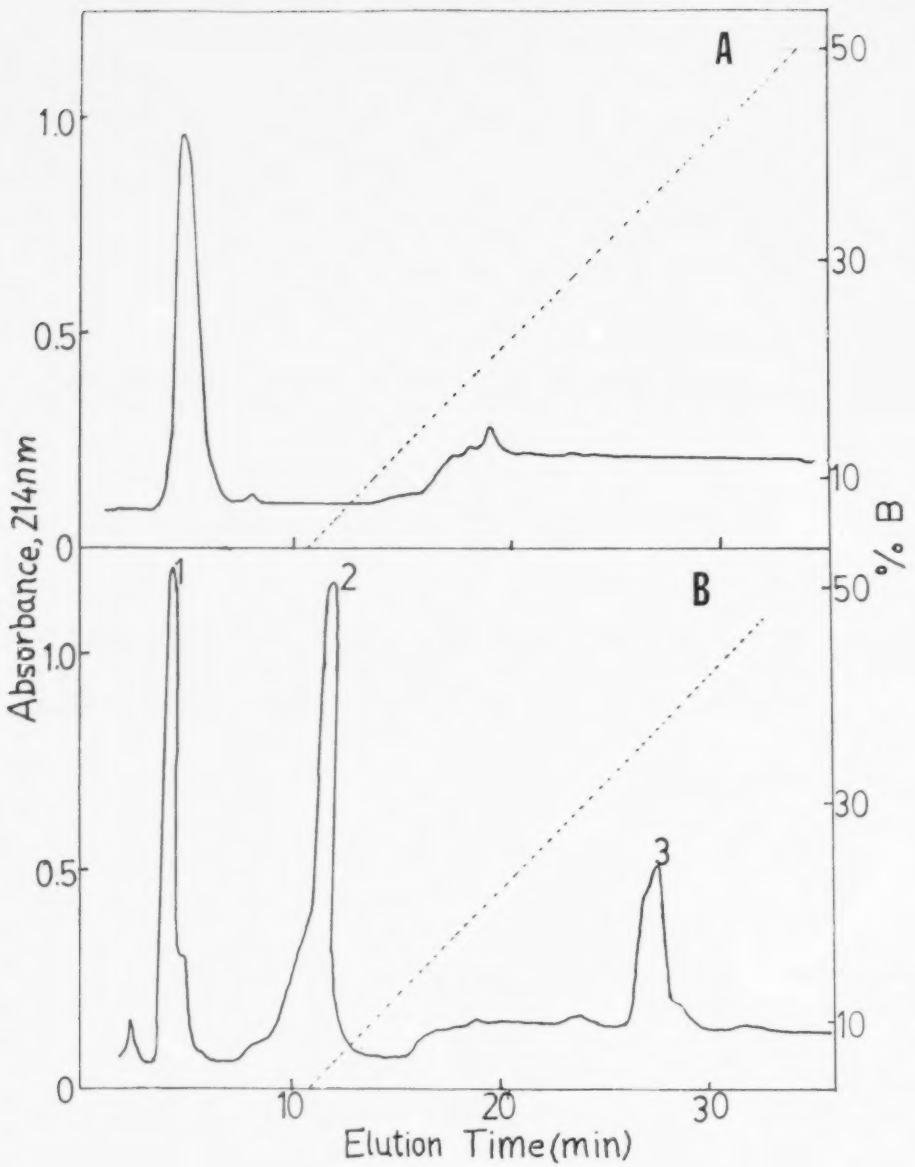


Figure 21. Strong cation exchange chromatography of Ac-PQPPQEKAHDGGRYYNH-$_2$ (α_1C-telo-2) digested with purified telopeptidase. Panel A:control peptide, B: 120 μL of digest. Column: Synchropak S300 (25 x 0.46 cm); Eluent A was 5mM KH_2PO_4, pH 6.5 and eluent B was 5mM KH_2PO_4, 0.5 M NaCl pH 6.5. A flow of 1 mL/ min and a gradient of 2% B/min were used. Fractions under peaks 1, 2 and 3 were individually pooled, dried and desalted on RP-HPLC before analysing.

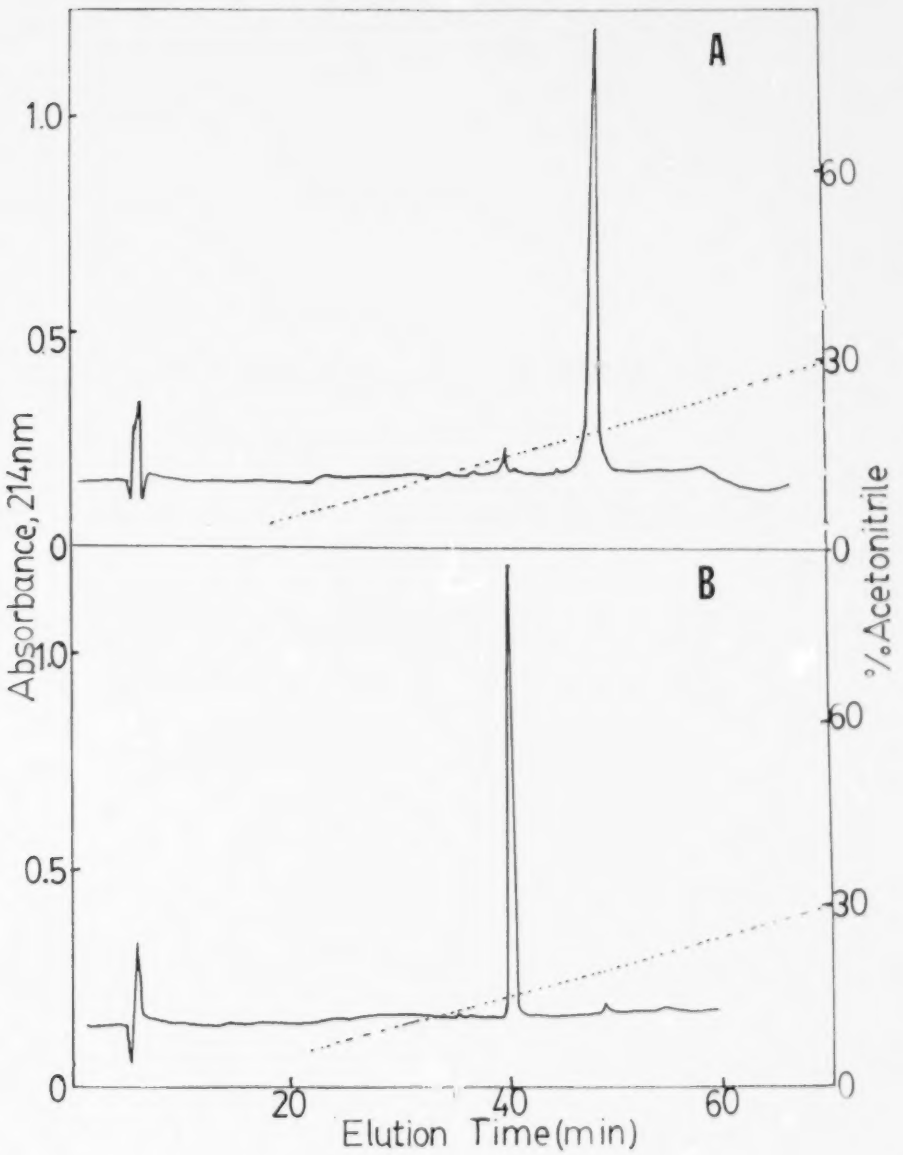


Figure 22. RP-HPLC chromatography of fraction 2 (Panel A) & 3 (Panel B) off cation exchange column. Column conditions were similar to those in Fig 19.

The peptide Ac-PQPPQE-COOH (or part of this peptide in case of multiple cleavage) was not detected. The fractions under the peak that eluted at 4 % B (peak 2) when run on RP-HPLC could not be detected probably due to low absorbance at the wavelength used. Another possible problem of using this two step method for purification was that the small amount of sample could have been lost on the column.

Table 4

4. Peptides of α_1C-telo-2 by telopeptidase 2 on cation exchange HPLC

Peak #	% B on CXC	Amino acid composition	Peptide
1	0	P Q P P Q E K A H 0.9,1.0,0.9,0.9,1.0,1.0,0.9,0.9,1.0 D G G R Y Y 1.0,1.0,1.0,1.1,1.1,1.1	undigested peptide
2	4	ND[1]	ND[1]
3	35	K A H D G G R Y Y 0.9,0.9,1.0.1.0,1.0,1.0,1.1,1.1,1.1	KAHDGGRYY-NH_2

1 *not detected on RP-HPLC*
CXC - *Cation exchange chromatography*

C.3.3. Separation of the Digested Fragments on RP-HPLC using Heptafluorobutyric acid as an Ion-pairing Agent

The fractions under the peaks eluting at 14.5 % B (1), 24.5 % B (2), and 26.5 % B (3) were pooled, dried and analyzed (Fig 23 panel B). Table 5 shows the amino acid composition of the fraction analysed.

Table 5

5. Peptides of α_1C-telo-2 by telopeptidase 2 on RP-HPLC using HFBA

Peak #	% B	Amino Acid Composition	Peptide
1	14.5	P Q P P Q E 1.1,0.9,1.1,1.1,0.9,0.9	Ac-PQPPQE-COOH
2	24.5	P Q P P Q E K A 1.1,1.1,1.1,1.1,1.1,1.1,0.9,1.0 H D G G R Y Y 0.9,1.1,1.1,1.1,0.9,0.9,0.9	Ac-PQPPQEKAHDGGRYY-NH_2
3	26.5	K A H D G G R Y Y 0.9,1.0,1.1,0.9,1.1,1.1,1.2,0.9,0.9	KAHDGGRYY-NH_2

The peak 1 that eluted at 14.5 % B gave the analysis of AcPQPPQE-. The uncleaved substrate, peak 2 eluted at 24.5 % B and -KAHDGGRYYNH$_2$, peak 3 eluted at 26.5 % B. Heptafluorobutyric acid is a more hydrophobic ion pairing agent than TFA and retains positively charged peptides longer on the RP-HPLC column. Hence the cleavage product KAHDGGRYY-NH_2 is retained longer than the uncleaved peptide, unlike the result when TFA was used (Fig 20).

To confirm that the peak eluted at 14.5 % B was intact Ac-PQPPQE-COOH and not a fragment of it with the same analysis (Pro:Glx, 1:1), authentic Ac-PQPPQE was added to the digest as an internal standard (Fig 23 Panel C). Since the absorbance where Ac-PQPPQE eluted increased considerably it was confirmed that the amino acid analysis shown by fraction at 14.5 % B in the digest was indeed that of the intact peptide Ac-PQPPQE and not fragments of it.

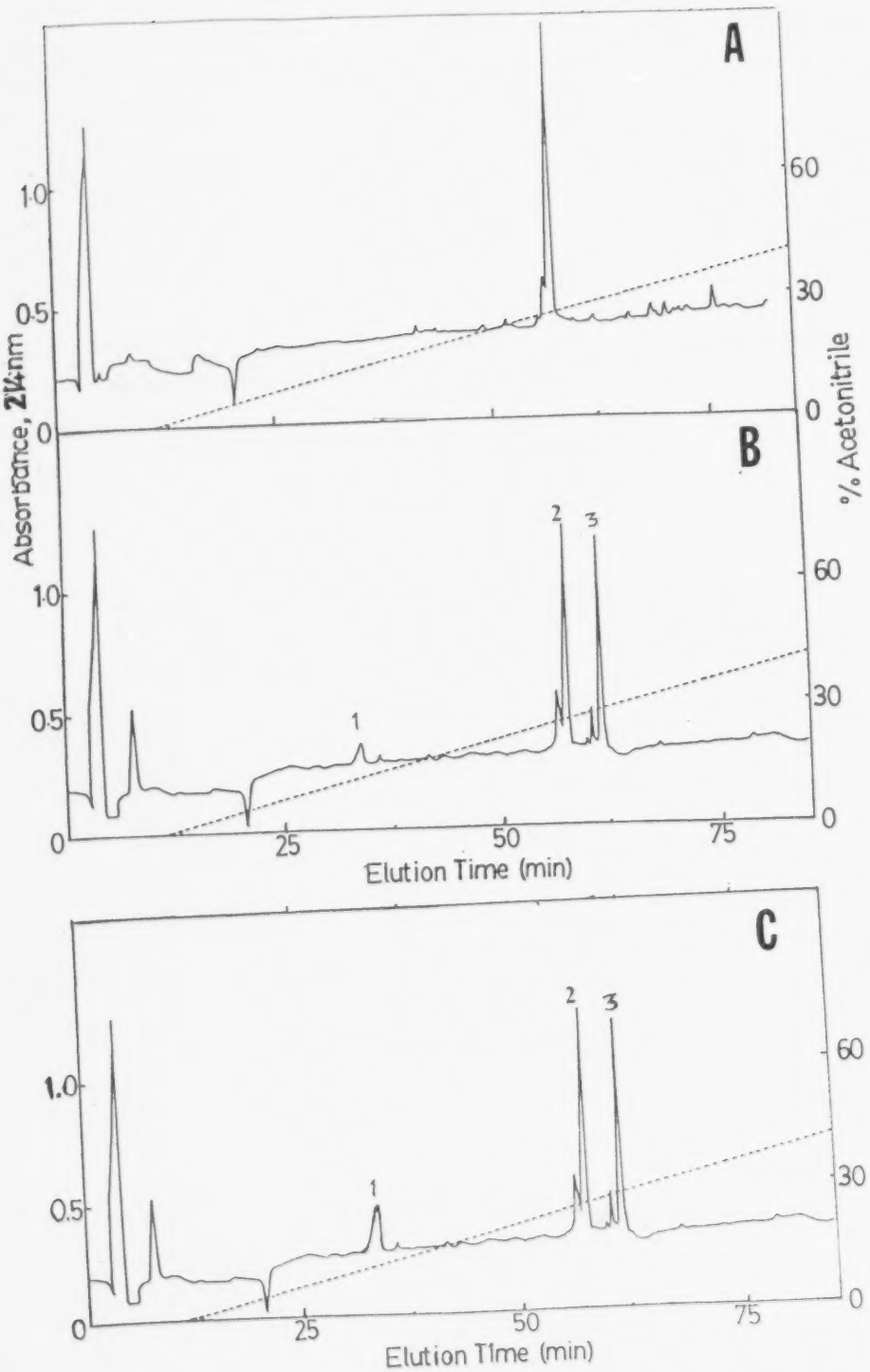
C.4. Action of Purified Telopeptidase 2 on Ac-GPPSGGYDLSFL-NH_2 ($N\alpha_1C$-telo)

The control peptide eluted at 30 % B when run on Vydac C_{18} RP-HPLC with 0.1% TFA (Fig 24). The digest run shows peaks eluting at 13.5, 18 and 25 % B in addition to the undigested substrate which eluted at 30 % B. Peak 1 was identified as Ac-GPPSGGYD-, 3 as Ac-GPPSGGYDL- and 4 as -LSFL-NH_2. Peak 2 was insufficient for analysis but is believed to be -SFL-NH_2 based on the known retention time of -FL-NH_2 under similar conditions (Results section E). The absorbance of the fragment -FL-NH_2 is very low at 214 nm since it has only two amide bonds.

Thus the scissile bonds for purified telopeptidase 2 can be summarized as:-

$N\alpha_1C$-telo	Ac-GPPSGGYD$\downarrow$L$\downarrow$SFL-NH_2
α_1C-telo-2	Ac-PQPPQE$\downarrow$KAHDGGRYY-NH_2

Fig 23. RP-HPLC chromatography of α_1C-telo-2 digested with telopeptidase 2 using HFBA as ion-pairing agent. Panel A: Control. Panel B: 120 μL of digest. Eluent A = 0.1% HFBA in water and B = 0.1% HFBA in acetonitrile. Flow was 1 mL/min and isocratic in A for 10 min, followed by a gradient of 0.5% B/min. Peak 1 was found to consist of PQPPQE and 3 was KAHDGGRYY. Panel C: Chromatogram of the digest to which Ac-PQPPQE-COOH was added.


A
B
C
1.0
0.5
0
60
30
0
Absorbance, 214nm
% Acetonitrile
1
2
3
25
50
75
Elution Time (min)

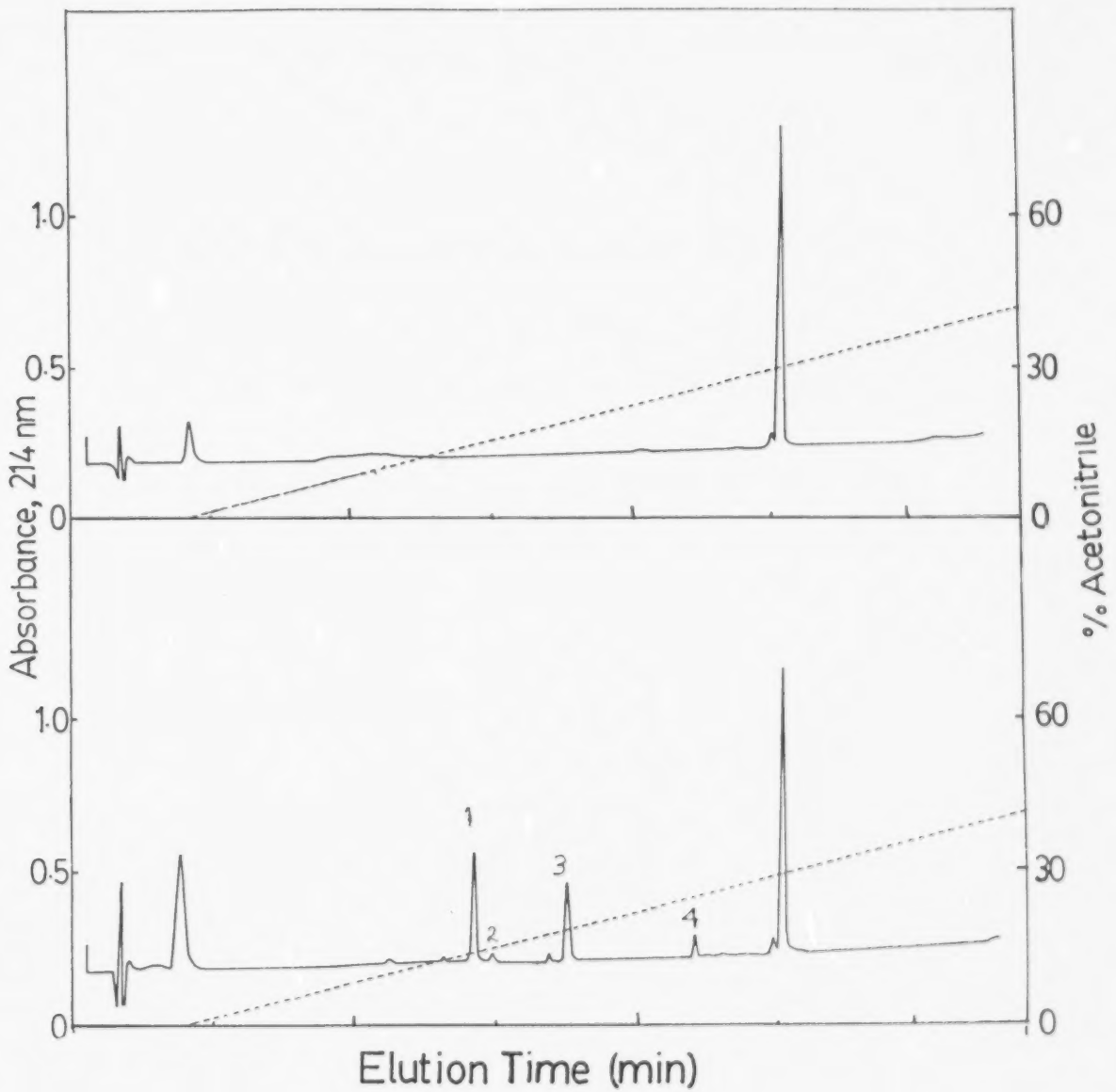


Figure 24. RP-HPLC chromatogram of $N\alpha_1$C-telo digested with purified telopeptidase. Panel A: control $N\alpha_1$C-telo, B: digest. Eluent A: 0.1% aqueous TFA, B: 0.1% TFA in acetonitrile. Flow 1 mL/ min, A gradient of 0.5 % B/min was applied after eluting isocratically with A for 10 min. Other details are as in Fig 19.

D. Determination of Scissile Bonds for Stromelysin

The amount of stromelysin /MMP-3 necessary was determined by a telopeptidase assay. Incubations were done according to the procedure described in the methods section B.9. The results of incubating the synthetic peptides with telopeptidase and MMP3 are summarized in Table 6:

Purified and recombinant MMP3 show similar effects on α_1C-telo-2. The cleaved fragment AcPQPPQE- eluted at 14.5 % B, -KAHDGGRYYNH_2 at 26.5 % B and the uncleaved substrate at 24.5 % B. The cleavage was thus between the Glu-Lys bond the result being the same as that of telopeptidase. Both purified and recombinant stromelysin cleaved AcGPPSGGYDLSFLNH_2 (Nα_1-Ctelo) between Asp and Leu. Purified stromelysin also showed a minor cleavage between Leu-Ser bond. In the digest of Nα_1C-telo digested with recombinant stromelysin, this minor cleavage was not seen. The digestion was weak.

Thus the enzyme has two scissile bonds in the C-terminal telopeptide of type I collagen, the major cleavage being between Glu & Lys, and the minor one between Asp & Leu.

Table 6

Comparison of Synthetic Substrate Cleavage by Telopeptidase 2, Purified and Recombinant MMP-3

Substrate	Substrate nmoles	Hours of Digestion	Tpase Units	Fragments Produced	nmoles* by 1u in 24 hours
Telopeptidase 2					
Nα,C-telo	14.6	64.5	0.065	Ac-GPPSGGYD- -LSFL-NH_2	ND 15.05
				Ac-GPPSGGYDL- -SFL-NH_2	6.1 ND
α,C-telo-2	5.44	53.0	0.069	Ac-PQPPQE- -KAHDGGRYY-NH_2	12.92 22.58
Purified MMP-3					
Nα,C-telo	8.32	80.0	0.15	Ac-GPPSGGYD- -LSFL-NH_2	3.52 4.56
				Ac-GPPSGGYDL- -SFL-NH_2	0.52 ND
α,C-telo-2	6.5	58.0	0.03	Ac-PQPPQE -KAHDGGRYY-NH_2	12.53 8.1
Recombinant MMP-3					
Nα,C-telo	16.3	40.0	0.23	Ac-GPPSGGYD- -LSFL-NH_2	2.85 ND
α,C-telo-2	8.16	40.0	0.23	Ac-PQPPQE- -KAHDGGRYY-NH_2	7.15 6.81

Nα,C-telo = AcGPPSGGYDLSFL-NH_2, α,C-telo-2 = AcPQPPQEKAHDGGRYY-NH_2
ND = Not Determined *Based on amino acid analysis.

E. Determination of the Scissile Bonds for Collagenase

The CNBr fragment of collagen, digested with the purified MMP3 sample that was contaminated with collagenase showed an increase in the α_1CB6c band (see section B.2., Fig 17C & 17D.). This implies that collagenase might be capable of cleaving the C-telopeptide. Hence purified MMP-1 (purified fraction 1 off chromatofocussing) was incubated with Nα_1C-telo and the fragments separated on RP-HPLC (Figure not shown). This peptide was cleaved by collagenase. The major cleavage being at the Ser-Phe peptide bond and a minor cleavage at the Asp-leu peptide bond. The cleavage between the Asp & Leu was also shown by telopeptidase. Although the Ser-Phe peptide bond was not cleaved by telopeptidase there is always the possibility that some telopeptidase may have eluted along with collagenase on chromatofocusing.

The RP-HPLC profiles of the two synthetic substrates digested with recombinant MMP-1 at 37°C are shown in Figures 25 and 26. The results of the 37°C digest are summarized in Tables 7 and 8.

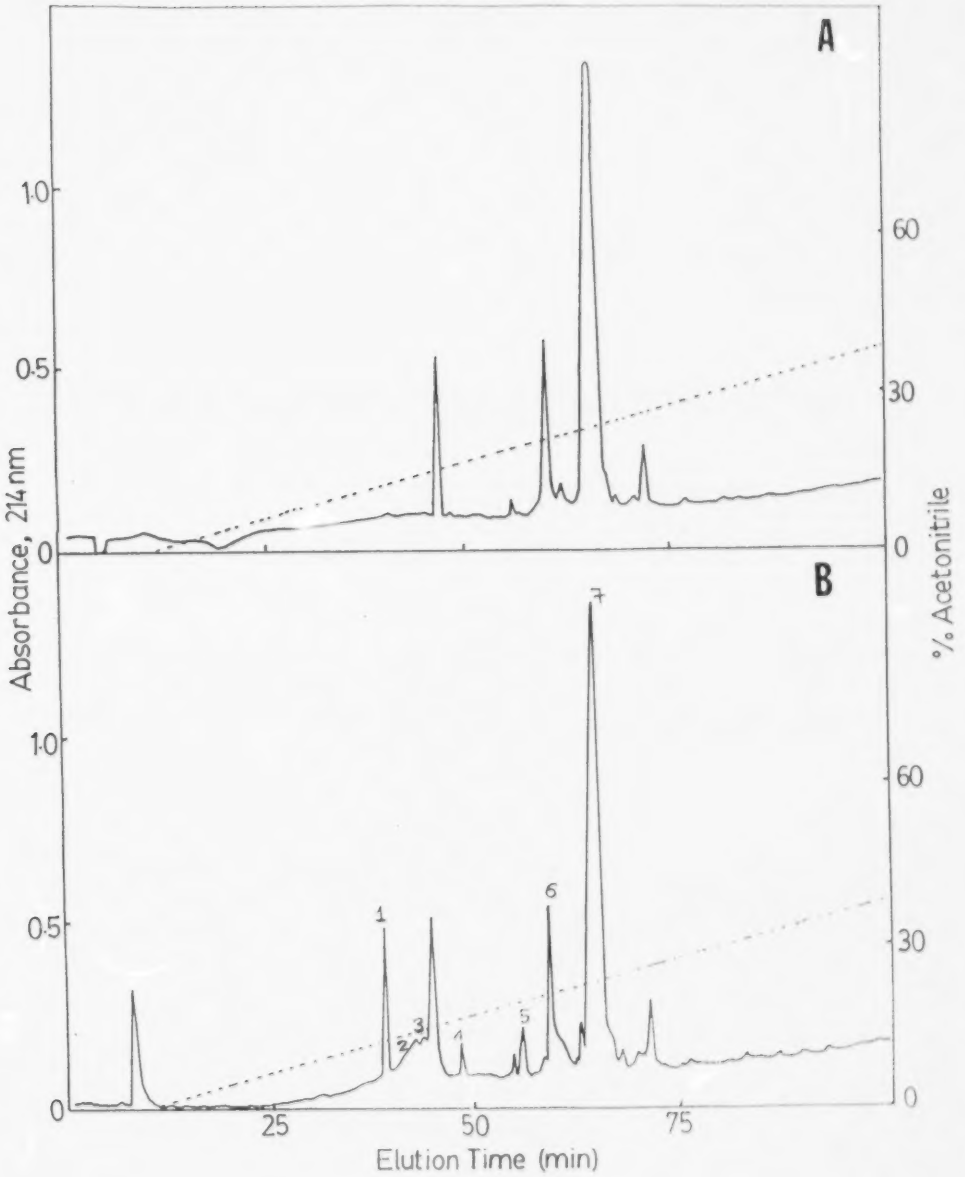


Figure 25. RP-HPLC of α_1C-telo-2 digested with recombinant MMP1. Buffer A = 0.1% HFBA in water & B = 0.1 % HFBA in acetonitrile. Panel A: control peptide; Panel B:digest. The column and conditions were as described for Fig 19. Fractions corresponding to each peak were pooled, dried and analyzed. Only the peaks found on analysis to contain peptide(s) are labelled.

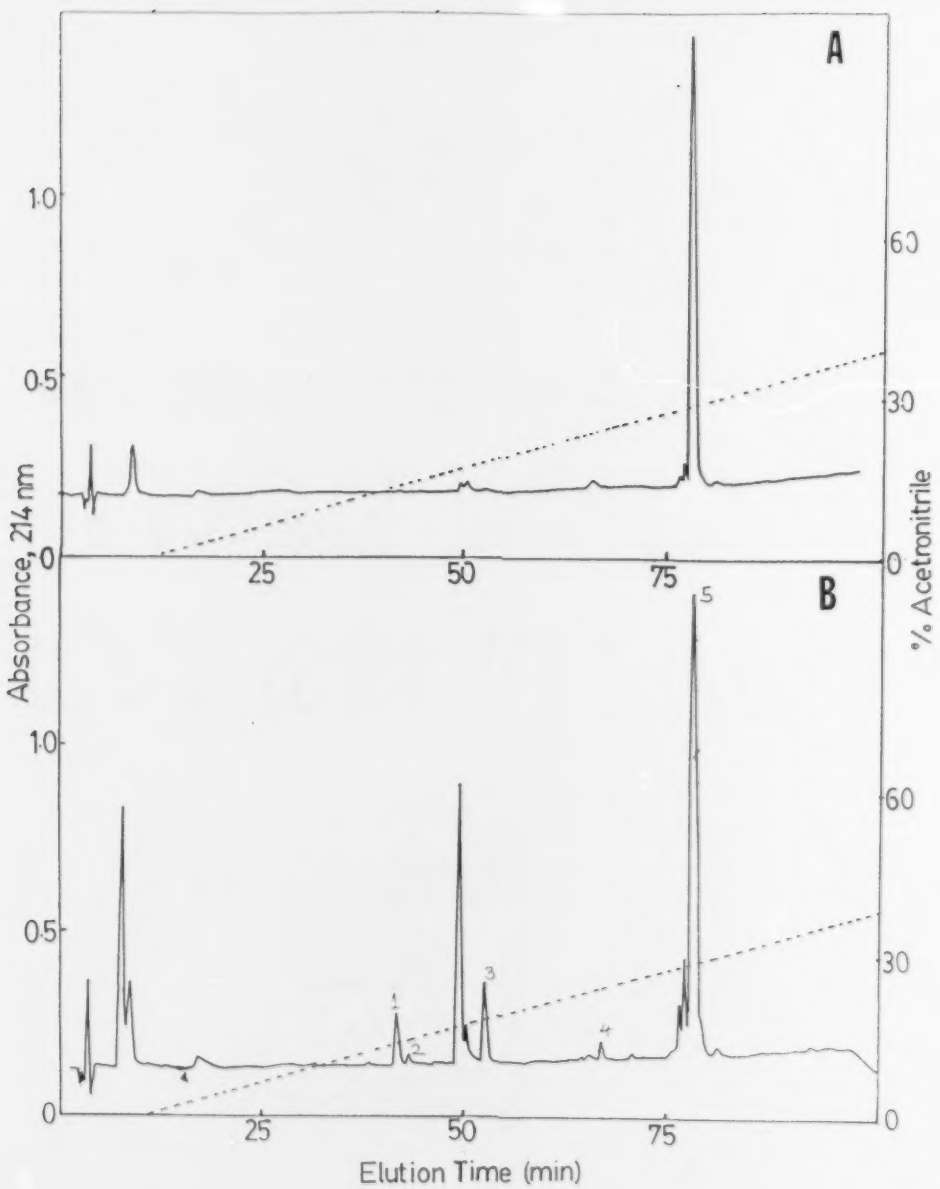


Figure 26. RP-HPLC of Nα,C-telo digested with recombinant MMP1. Panel A: control peptide; panel B: digest. The column and conditions were as described for Fig 19. Peaks 1 = Ac-GPPSGGYD-, 2 = -FL, 3 = Ac-GPPSGGYDLS, 4 = LSFL and 5 = Ac-GPPSGGYDLSFL (uncleaved substrate).

Table 7

7. Peptides Produced by Action of Recombinant MMP-1 on α_1C-telo-2

HPLC peak #	Peptide	% B	nmoles of peptide in fraction
1	Y	13.0	1.81
2	PQPPQEK	14.75	1.37
3	PQPPQE	15.0	0.66
4	K	16.75	0.23
5	AHDGGRY	20.5	2.08
6	Ac-PQPPQEKAHDGGRY	21.5	0.98
7*	Ac-PQPPQEKAHDGGRYY-NH_2	25.25	1.5

Table 8

8. Peptides Produced by Action of recombinant MMP-1 on Nα_1C-telo

HPLC peak #	Peptide	% B	nmoles of peptide in fraction
1	Ac-GPPSGGYD	13	0.67
2	-FL-	14	0.92
3	Ac-GPPSGGYDLS	18	1.57
4	-LSFL-NH_2	24	1.1
5*	Ac-GPPSGGYDLSFL-NH_2	29	61.0

* - undigested substrate

Thus the cleavage by MMP-1 on $N\alpha_1$C-telo and α_1C-telo 2 can be summarized as:

$N\alpha_1$C-telo	Ac-GPPSGGYD $\downarrow$ LS $\downarrow$ FL-NH_2
α_1C-telo-2	Ac-PQPPQE $\downarrow$ K $\downarrow$ AHDGGRYYNH_2

Several attempts were made to show that recombinant MMP-1 cleaves acid soluble collagen in the α_1CB6 region but due to the large amount of α_1CB7' produced by the collagenolytic activity of recombinant MMP-1 and the slight difference in the molecular weights of α_1CB7' and α_1CB6, they could not be resolved. Since in fibrillar collagen Lys_{17} is cross-linked, the cleavage before and after lysine may not be easily accessible for the MMPs in order to bring about the destruction of collagen fibrils. Thus the cleavage(s) in the -GGYDLSFL- sequence may be the first target by one or more of these MMPs during the destruction of collagen.

F. Ac-PQPPQE-COOH as Competitive Product Analog of Collagen

At 1.3 nmoles Ac-PQPPQE-COOH did not show any inhibition of telopeptidase activity, but at 148 nmoles concentration a 47.46% inhibition of the telopeptidase activity was observed.

G. Design of Assay using Synthetic Peptide for Telopeptidase 2 / MMP-3 Activity

The digest of peptide while on resin when run on RP-HPLC showed a few

small peaks. These when analyzed did not show any cleavage products.

The digested sample of the soluble peptide when run on RP-HPLC gave peaks eluting at 19.5, 22, 23.5, 29.5 %B, which were hydrolyzed with 6N HCl for 24 hrs. at 110°C. Amino acid analysis showed:

19.5 %B	no peptide	
22.0 %B	KAHD-NH_2	3.8 nmoles
23.5 %B	Z-PQE	3.4 nmoles
29.5 %B	Z-PQEKAHD-NH_2	41 nmoles

In conclusion the enzyme did not cleave the above peptide resin possibly due to steric reasons, the cleavage site being next to the lysine attached to the bulky resin. In order to set up a sensitive assay it is important to use a substrate with a low K_m or a high k_{cat}/K_m.

H. Inhibition Studies

H.1. Human Plasma Alpha$_2$-Macroglobulin as Inhibitor of Telopeptidase and Collagenase

No inhibition of telopeptidase activity in fibroblast culture medium was found at 0.056 and 0.112 nM of the inhibitor. Alpha-2-macroglobulin is known to inhibit enzymes belonging to all four classes. Hence it is posible that the concentration used in this assay was not sufficient to show any inhibition of telopeptidase. There was a 25% reduction in collagenase activity by 4.47 pmoles of α-2M.

H.2. Effect of Synthetic Inhibitors on Matrix Metalloproteinases

The concentration of inhibitor required to inhibit the enzyme activity by 50% (IC_{50}) was determined using a computer program (GraphPAD InPlot). Figure 27 shows a sigmoid plot obtained for inhibition of MMP-2 by Z-Pro-Leu-Gly-NHOH at a substrate concentration of 10μM.

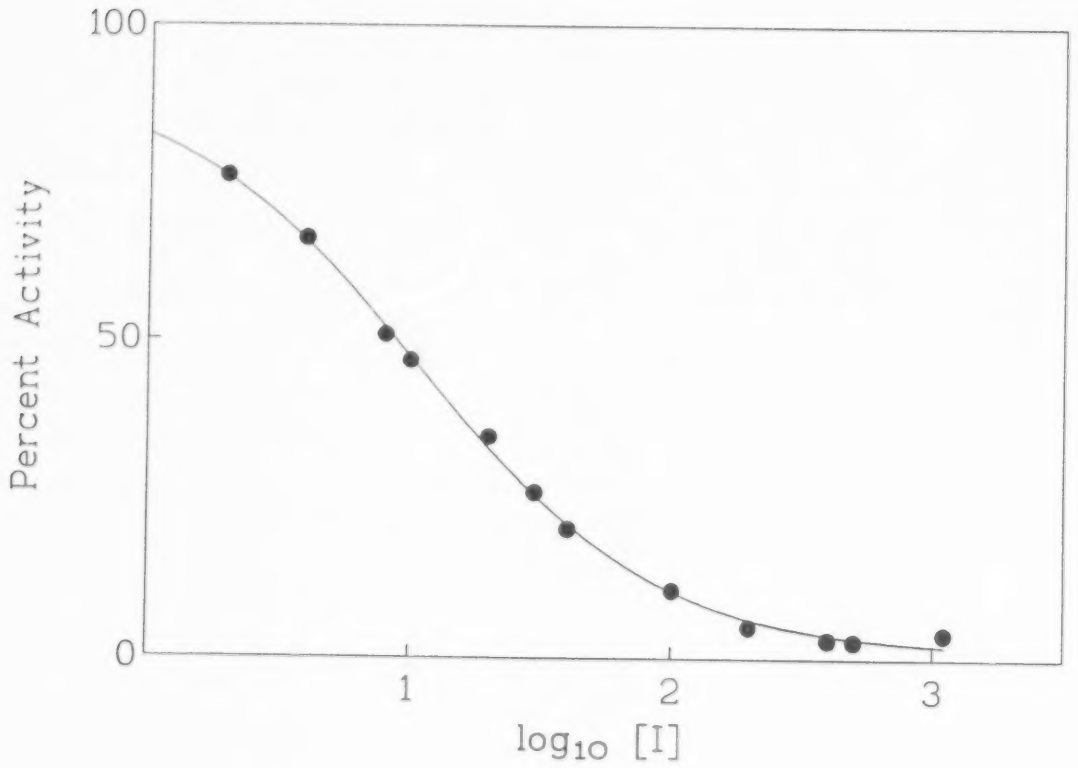


Fig 27. Plot of activity verses $\log_{10}$[I] μM for the determination of IC_{50} for Z-Pro-Leu-Gly-NHOH on MMP2 at a substrate concentration of 10μM.

Almost all of the inhibitors synthesized showed measurable inhibition of MMP-2 (Table 9). The hydroxamate group made a major contribution to the inhibitory potency of the Z- di- and tripeptides and is presumably acting as designed to chelate the zinc at the active site of the enzymes. The inhibition was weak when the free acid was tested (inhibitors 2 & 6 in comparison to 1 & 5). It is assumed that the binding of each of these inhibitors is to the $S_1....S_n$ subsites of the enzyme (see Fig 3.; Schechter and Berger, 1967). The substitution of Gly for Leu at the P_2 position increased the IC_{50} by nearly 10-fold (inhibitors 1 & 4) and 100-fold (inhibitors 5 & 9), suggesting that a hydrophobic residue at P_2 is favourable for binding. The presence of a free amino group on the peptides greatly increased the IC_{50} (inhibitors 3 & 7 in comparison to 1 & 5). This effect was most marked for the tripeptide (Z)-Pro-Leu-Gly-NHOH. Acetylation (Ac-Pro-Leu-Gly-NHOH) only partly restored the inhibitory potency of NH_2-Pro-Leu-Gly-NHOH, suggesting that a hydrophobic side-chain at P_4, provided by the Z- group is conducive to inhibition. This would be consistent with the observation that Pro-Pro-Leu-Gly-NHOH was almost as effective an inhibitor as Z-Pro-Leu-Gly-NHOH and much more effective than Pro-Leu-Gly-NHOH. Other evidence for the importance of the P_4 residue comes from the observation that Z-Gly-Pro-Leu-Gly-NHOH was a much weaker inhibitor than Z-Pro-Pro-Leu-Gly-NHOH and that Z-(D)-Pro-Pro-Leu-Gly-NHOH was four-fold weaker. At the P_4 subsite (L)-Pro is better when compared to Gly, (D)-Pro, L-Leu or the Z- group (inhibitors 13, 11, 14, 5), so that the size of side-chain that can be accomodated at the S_4 subsite of the enzyme must be limited. At the

S_5 subsite the Z group is better accomodated than a free amino group (10 and 12). It is not possible to comment on the nature of this subsite except that it either prefers a hydrophobic group or does not accept the charge on the amino group. While we can conclude that the substrate binding site of MMP-2 is quite extended and can accomodate at least five amino acid side-chains, the present data do not permit us to deduce the existence of an S_6 subsite.

TABLE 9

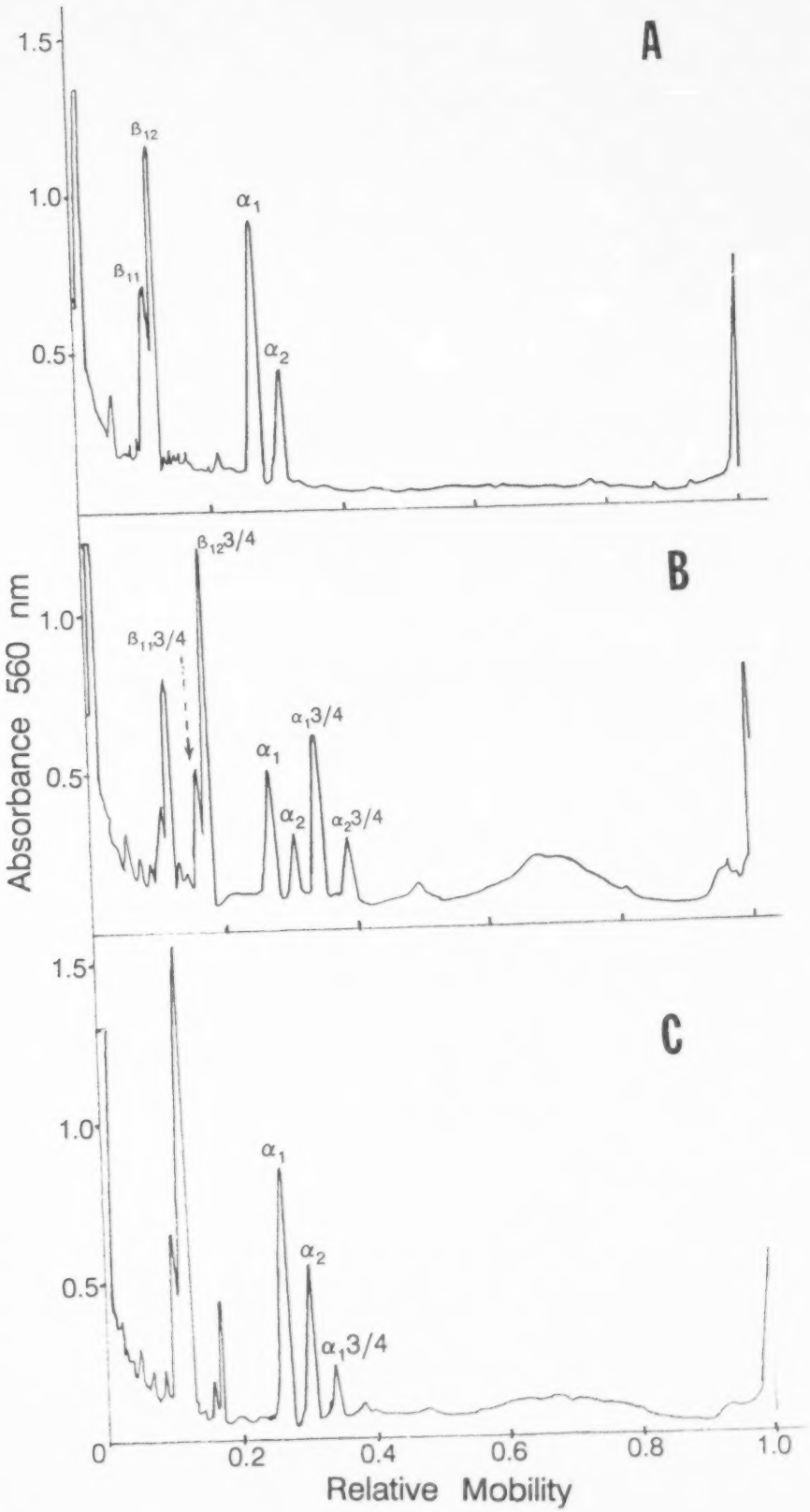
9. Comparison of IC_{50} of the Inhibitors of Matrix Metalloproteinase 2

Number	Inhibitor	IC_{50} (μM)
1	Z-Leu-Gly-NHOH	56.0
2	Z-Leu-Gly-COOH	5000
3	NH_2-Leu-Gly-NHOH	2900
4	Z-Gly-Leu-NHOH	120
5	Z-Pro-Leu-Gly-NHOH	16.0
6	Z-Pro-Leu-Gly-COOH	2700
7	NH_2-Pro-Leu-Gly-NHOH	1500
8	Ac-Pro-Leu-Gly-NHOH	75.7
9	Z-Pro-Gly-Gly-NHOH	1500
10	Z-Pro-Pro-Leu-Gly-NHOH	1.6
11	Z-(D)-Pro-Pro-Leu-Gly-NHOH	7.7
12	NH_2-Pro-Pro-Leu-Gly-NHOH	18.0
13	Z-Gly-Pro-Leu-Gly-NHOH	12.7
14	Z-Leu-Pro-Leu-Gly-NHOH	18.5

All assays were done at a substrate concentration of 0.10μM.

Some of these inhibitors were tested against collagenase/MMP-1 and MMP-3. Fig 28 shows the scans of acid soluble collagen digested with collagenase (Fraction 1 off chromatofocusing). Amount of enzyme used was such that approximately 50-70% digestion of collagen occurred in the absence of inhibitor. Inhibition by 0.25 mmoles of Z-Pro-Leu-Gly-NHOH is shown in Panel C. % inhibition was calculated as discussed in the methods. Similarly Fig 29 shows a range of inhibitor concentrations (Z-(L)-Pro-Pro-Leu-Gly-NHOH tested against collagenase. At 0.5 mM of inhibitor 93% inhibition occurred (gels 5 & 6), while 0.005 mM showed 20 % inhibition (gels 17 & 18). With decrease in inhibitor concentration the presence of $\alpha_1$3/4 and $\alpha_2$3/4 bands become significant (gels 5 to 18). Table 10 shows the IC_{50} and K_i values determined for inhibitors tested for MMP-1 and MMP-3. The same general trends were seen as for MMP-2, with the longer peptides being more effective. It can be tentatively concluded from these data that inhibitors tested with all three enzymes were effective in the order MMP-2 > MMP-3 > MMP-1. A rigorous quantitative comparison would depend on careful analysis of the mechanisms of inhibition and the determination of the relevant kinetic parameters. Cornish-Bowden plots (Cornish-Bowden; 1974) of inhibition data at two substrate concentrations give pairs of lines which converge towards an intersection point at negative values of [I] and [S/V]. This is interpreted to mean that the inhibition mechanism is predominantly competitive but with a small uncompetitive contribution (Knight 1986). All combinations of enzymes and inhibitors showed this effect (Fig 30). For strictly competitive inhibitors the lines would be parallel.

Figure 28. Scans of 5% SDS gel electrophoresis showing [A] control acid soluble collagen; [B] ASC digested with collagenase (Fraction 1 off chromatofocusing). Amount of collagenase used was such that 66% of the collagen was digested. The broad region in 0.6 to 0.8 relative mobility shows unresolved $\alpha_1 1/4$ and $\alpha_2 1/4$ fragments. [C] collagenase activity inhibited by Z-Pro-Leu-Gly-NHOH. Considering the digestion of ASC by collagenase in panel B as 100%, 0.25 mmoles of Z-Pro-Leu-Gly-NHOH showed 33% inhibition.


A
B
C
1.5
1.0
0.5
0
β_{12}
β_{11}
α_1
α_2
β_{12}3/4
β_{11}3/4
$\alpha_1$3/4
$\alpha_2$3/4
Absorbance 560 nm
0.2
0.4
0.6
0.8
1.0
Relative Mobility

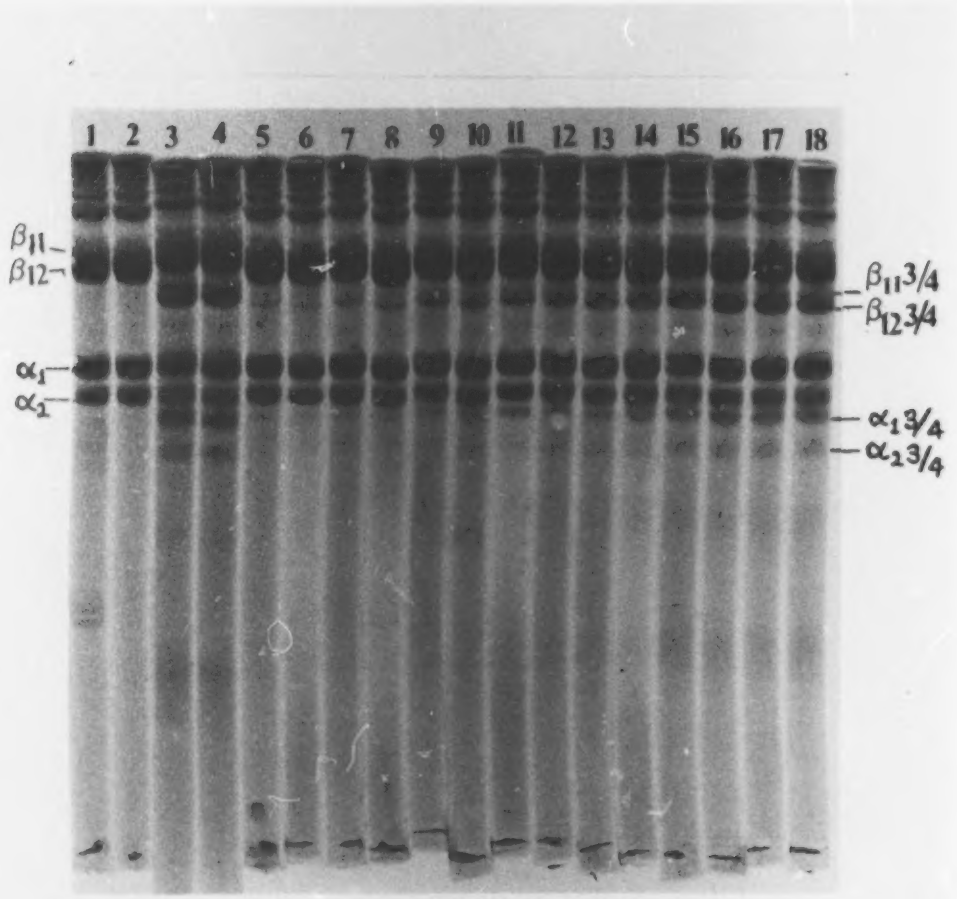


Figure 29. 5% SDS gel electrophoresis of ASC collagen. Gels 1 & 2 no enzyme controls, gels 3 & 4 enzyme control. Gels 5 to 18 show inhibition of collagenase activity on adding the inhibitor Z-(L)-Pro-Pro-Leu-Gly-NHOH at 0.5, 0.25, 0.1, 0.075, 0.05, 0.01 and 0.005mM concentrations respectively. Each concentration is in duplicates.

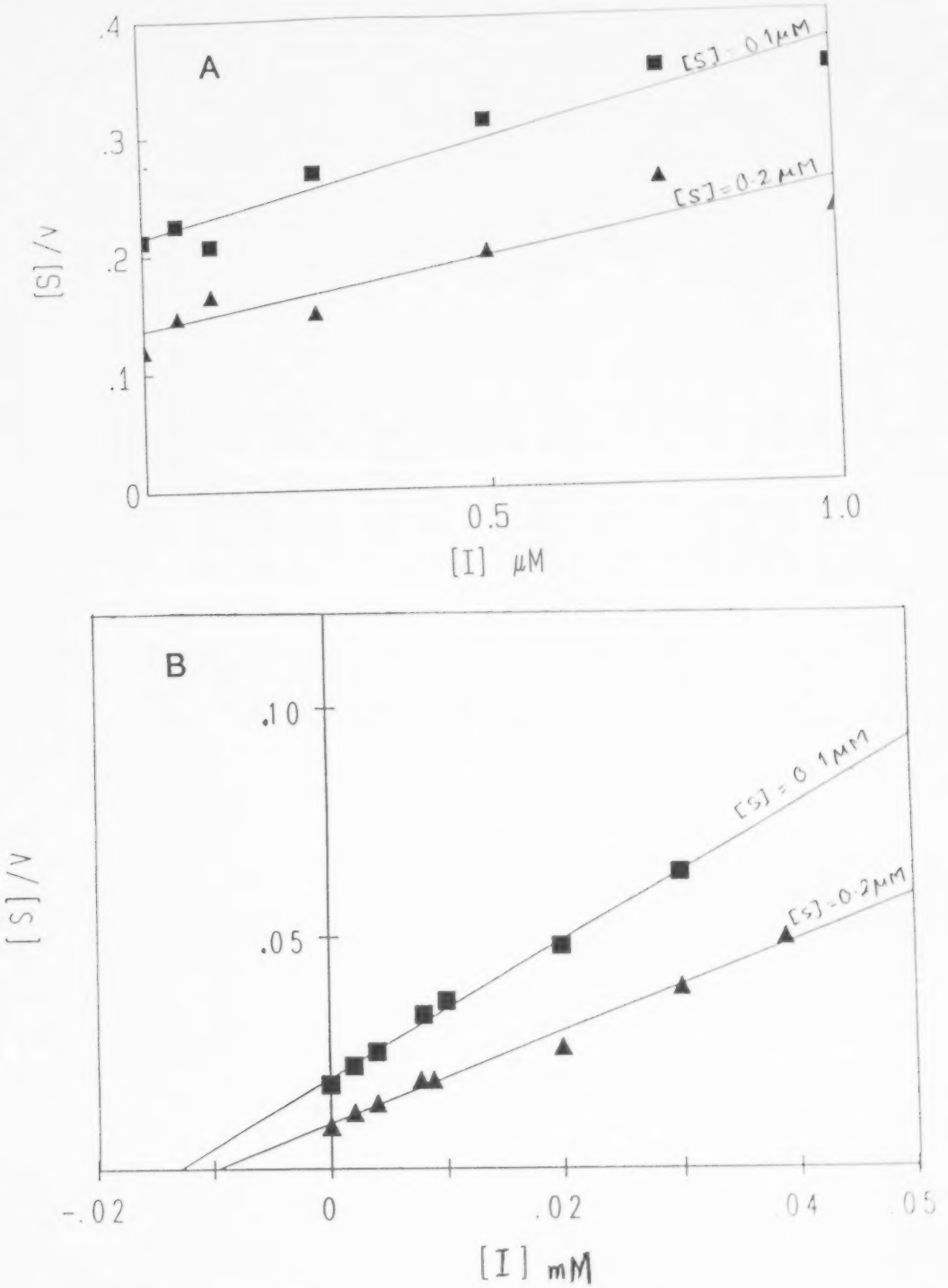


Figure 30. Cornish-Bowden plot for the inhibition of MMP-2 by Z-(L)Pro-Pro-Leu-Gly-NHOH (panel A), and of MMP-3 by Z-(L)Pro-Leu-Gly-NHOH (panel B). Substrate concentrations used were: ■ 0.1μM; and ▲ 0.2μM.

TABLE 10

10. Comparison of Inhibitors of Matrix Metalloproteinases 1 and 3

# from Table 1	Inhibitor	MMP-1[1] $IC_{50}(\mu M)$	$K_i(\mu M)$	MMP-3[2] $IC_{50}(\mu M)$	$K_i(\mu M)$
1.	Z-Leu-Gly-NHOH	340	ND	200	186
4.	Z-Gly-Leu-NHOH	1900	ND	ND	ND
5.	Z-Pro-Leu-Gly-NHOH	130	60	8.5	15
9.	Z-Pro-Gly-Gly-NHOH	2200	ND	ND	ND
10.	Z-Pro-Pro-Leu-Gly-NHOH	29	31	3.8	6.5
11.	Z-(D)-Pro-Pro-Leu-Gly-NHOH	75	ND	ND	ND
13.	Z-Gly-Pro-Leu-Gly-NHOH	57.7	ND	ND	ND

1 *[S] = 1.33μM, except for Z-Gly-Leu-NHOH ([S] = 0.95μM)*
2 *[S] = 1.21μM.*
ND *Not determined*

Dixon plot (1/V against [I] for two or more values of [S]) was used for determination of K_i. The K_i values could be obtained from Dixon plots for some combinations of inhibitor and enzyme. These are included in Table 10 for MMP-1 and MMP-3, the intersection was in the upper left quadrant for all inhibitors except Z-Leu-Gly-NHOH. Figure 31 shows a Dixon plot for Z-Pro-Pro-Leu-Gly-NHOH inhibiting MMP-2. For MMP-3, however, all intersections were in the lower left quadrant (not shown), indicating that the uncompetitive component was greater for this enzyme than for MMP-1 or MMP-2.

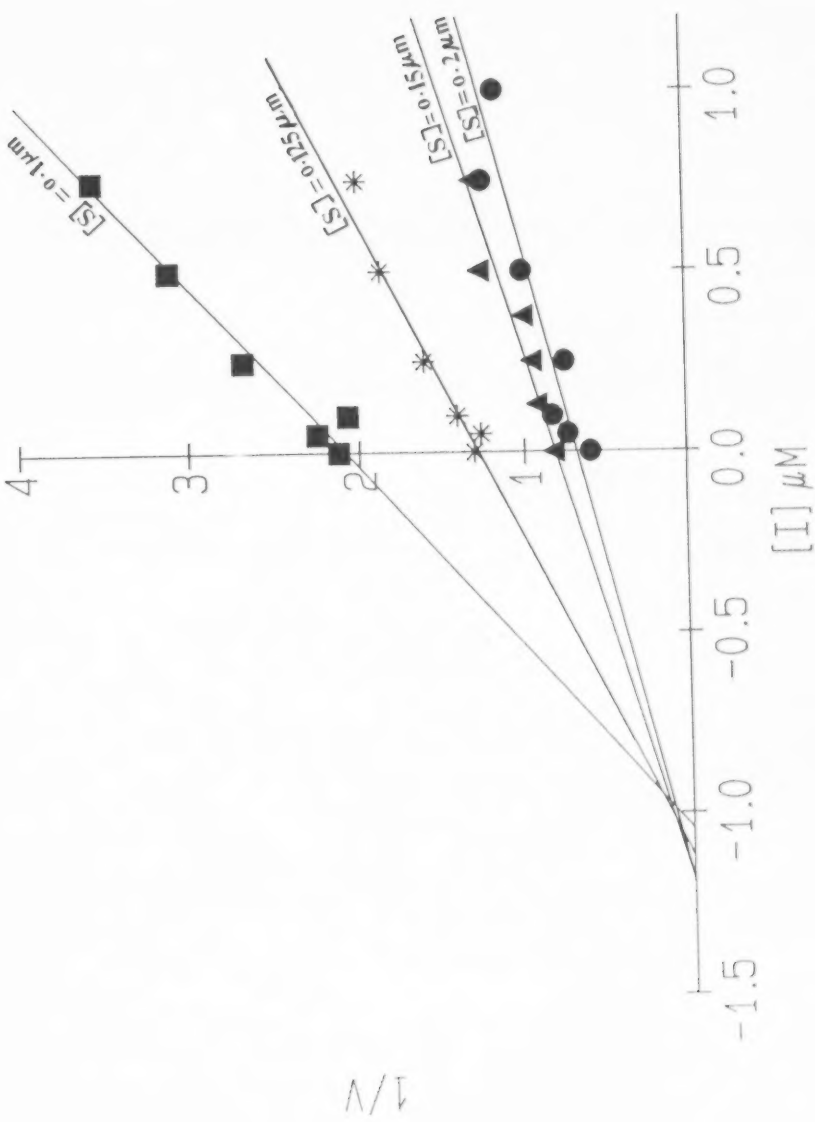


Figure 31. Dixon plot for inhibition of MMP-2 by Z-Pro-Pro-Leu-Gly-NHOH2. Substrate concentrations used were: ■ 0.1μM; ✱ 0.125μM; ▲ 0.15μM; ● 0.2μM.

Peptide-amides were synthesized using fmoc synthesis and screened for their inhibitory activity. The positions P-3 and P-4 were kept constant since this sequence (Pro-Pro) is present in the substrate. The best hydroxamate inhibitor was Z(L)-PPLG-NHOH, therefore the sequence Z(L)-PPL- was kept constant and at position P-1, K (+charge), D & E (- charge), L (hydrophobic residue), C (amino acid with -SH group), Q and G were synthesized. The peptide with Gly at position P-1 was synthesized in order to compare with the Z-Pro-Pro-Leu-Gly-NHOH previously tested. At position P-2; Q was synthesized since in the scissile bond PQPPQE-KAHD-, with G at P-1 to compare with Z-PPLG-NHOH (already tested) and with E at P-1 because sequence is similar to that in the substrate. The IC_{50} results are listed in Table 11.

The inhibition of MMP-3 by all the amides was weak and high concentrations had to be used. The peptides were insoluble at concentrations higher than 6 mM. Z-Pro-Pro-Leu-Leu-NH_2 was insoluble even at lower concentrations and hence no data could be obtained. It was not possible to calculate the IC_{50} using the computer program, GraphPAD InPlot™ for MMP-3 because a wide range of concentrations, which is necessary to get a sigmoid plot, was not available. Z-Pro-Pro-Gln-Glu-NH_2 showed no significant inhibition of MMP-3 and Z-Pro-Pro-Leu-Gly-NH_2 inhibited its activity only by 14% at 5 mM concentration. The inhibitor Z-Pro-Pro-Gln-Glu-NH_2 was a weaker inhibitor than Z-Pro-Pro-Leu-Gly-NH_2.

The inhibition of gelatinase by these amides was better. At position P_1 the presence of an acidic, basic or a neutral residue did not make significant difference

in the inhibition of MMP-2, although a hydrophobic residue (Leu) as compared to a Gly at this position decreased the inhibition. The IC_{50} for Z-Pro-Pro-Gln-Glu-NH_2 was 5.59 mM as compared to 1.1 mM for Z-Pro-Pro-Leu-Gly-NH_2. Z-Pro-Pro-Leu-Asp-NH_2 showed the strongest inhibition of MMP-2 (IC_{50} of 0.7 mM) among the amides tested.

Table 11

11. Comparison of Z-peptide-NH_2 inhibition on MMP-2 & MMP-3

Peptide	MMP-3	IC_{50} for MMP2
Z-PPQG-NH_2	No inhibition upto 5 mM	3.8 mM
Z-PPQE-NH_2	No inhibition upto 5 mM	5.59 mM
Z-PPLK-NH_2	17% inhibition at 6 mM	0.92 mM
Z-PPLD-NH_2	No inhibition upto 4 mM	0.7 mM
Z-PPLQ-NH_2	IC_{50} = 3.58	0.97 mM
Z-PPLG-NH_2	14% inhibition at 5mM	1.1 mM
Z-PPLC-NH_2	ND	No inhibition at 4 mM
Z-PPLL-NH_2	ND[1]	ND[1]
Z-PPLE-NH_2	ND	7 % inhibition at 4 mM

1 Peptide was insoluble

ND not determined

I. Effect of Inhibitors on the Digestion of Collagen Fibrils by Fibroblasts

The FITC-labelled fibrillar collagen was completely converted into soluble peptides by bacterial collagenase in 1 hour at 37°C. In this period only 11% as much fluorescence was released by trypsin. Inspection under the fluorescnce

microscope confirmed the complete loss of fibrillar collagen from plates incubated with bacterial collagenase and no visible change in the plates incubated with trypsin. Even at 24 hours trypsin had caused the release of only 16% of the total fluorescence (determined by measuring the residual fluorescence released by collagenase and substracting the control value). The labelling and processing of the fibrillar collagen therefore had not led to significant degree of denaturation, which would have rendered it susceptible to digestion by trypsin.

Reversed-phase HPLC of the products of digestion of FITC-labelled collagen with bacterial collagenase (Figure 32) showed a complex pattern of peptides, several of which demonstrated measurable fluorescence. The amino acid compositions of three of these peptides were determined and in each case a content of about 10% of hydroxyproline was found. These results establish that the collagen had been labelled by covalent modification with FITC.

Progressive digestion of the fluorescent collagen fibrils by the fibroblasts could be followed by measuring the appearance of fluorescence in the medium. Visual confirmation of the process could be obtained by examining the wells under the fluorescence microscope (Fig 33). This revealed that digestion proceeded through the fragmentation and dissolution of the collagen fibrils. The release of fluorescence into the medium closely paralleled the release of hydroxyproline-containing fragments.

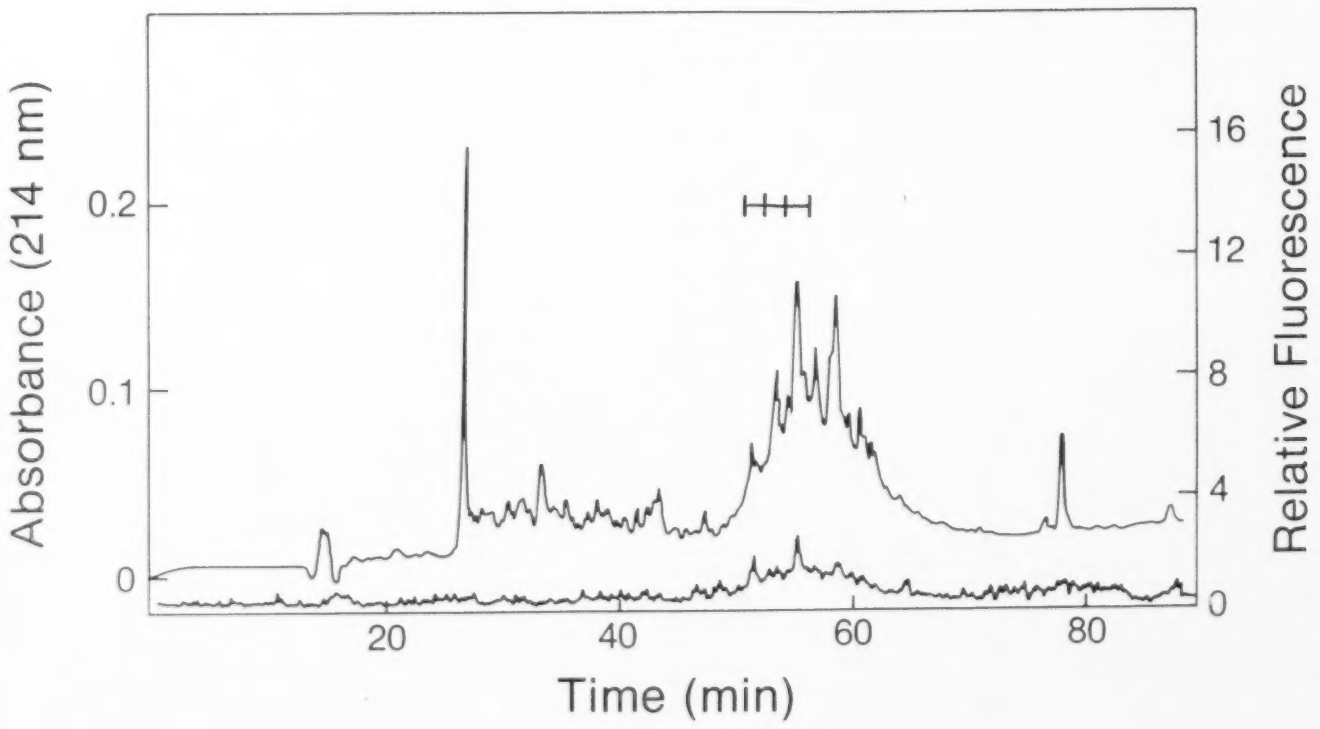


Figure 32. Reversed-Phase HPLC of peptides released by digestion of FITC-collagen with bacterial collagenase. An aliqout (0.5 mL) of supernatant was injected onto a 10 mm x 250 mm C18 column (Vydac 218TP1010; Elution was performed at 1 mL/min with a linear gradient of acetonitrile (1%/minute, starting at 10 minutes) in 0.1% (v/v) trifluoroacetic acid. Effluent was monitored at 214 nm (upper trace) and by fluorescence using a Gilson Spectra-Glo filter fluorometer (λ_{ex} 330-440 nm, λ_{em} 460-600 nm) (lower trace). Bars denote fractions which were pooled for amino acid analysis.

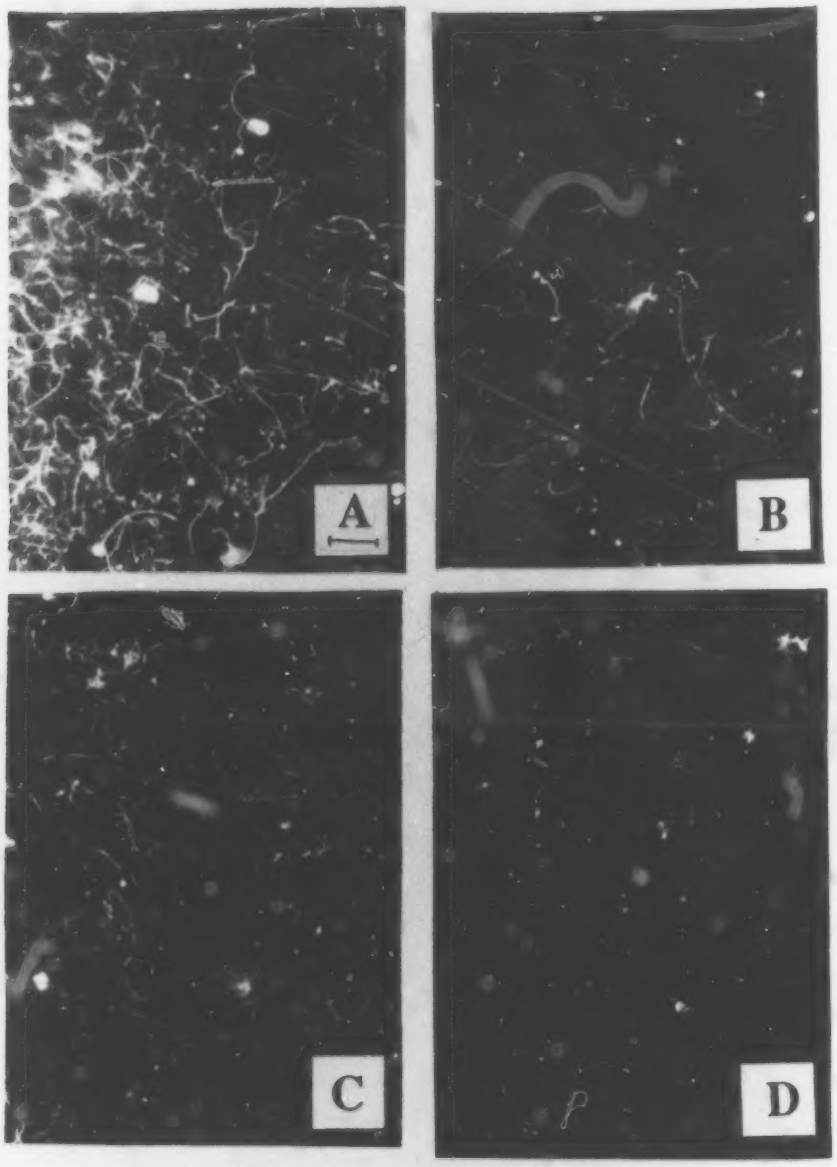


Figure 33. Visual demonstration of the progressive digestion of fluorescent fibrillar collagen by human gingival fibroblasts. Cells were seeded at high density (5 x 10^5) in a 6-well plate in DMEM-10 and allowed to reach confluence (1 day). The medium was changed to DMEM-0 and the plates were photographed in the fluorescence microscope 0 (A), 2 (B), 4 (C) and 6 (D) days later. The bar indicates 100 μm.

Human skin and gingival fibroblasts were grown on plastic multiwell plates coated with FITC-labelled fibrillar collagen. In the absence of serum, collagen is degraded to small peptides which can be detected in the culture medium by the fluorescence released (serum contains inhibitors such as $\alpha_2$macroglobulin and TIMP that inhibit MMPs). Different concentrations of the inhibitor were added to triplicate wells and the accumulated fluorescence plotted over a number of days (Fig 34). A decrease in the accumulated fluorescence corresponding to increased inhibition with increased concentrations of inhibitor was seen. The synthetic inhibitor Z-Pro-Leu-Gly-NHOH markedly reduced the rate of release of fluorescence into the medium by both gingival (Figure 34) and skin fibroblasts (not shown). At high concentrations (200 μM) it abolished collagen degradation by the strain of gingival fibroblasts tested and reduced it by about 80% in the particular strain of skin fibroblasts tested. This study suggests that compounds of this type are able to at least partially inhibit the destruction of fibrillar collagen by fibroblasts *in vitro*. To test for the viability of the cells in the presence of the inhibitors, the fibroblasts on the cell culture plates were briefly trypsinized and counted. The cell counts before and after the inhibition experiments were constant. Hence the inhibitors at the concentrations at which they were used were not toxic to the cells.

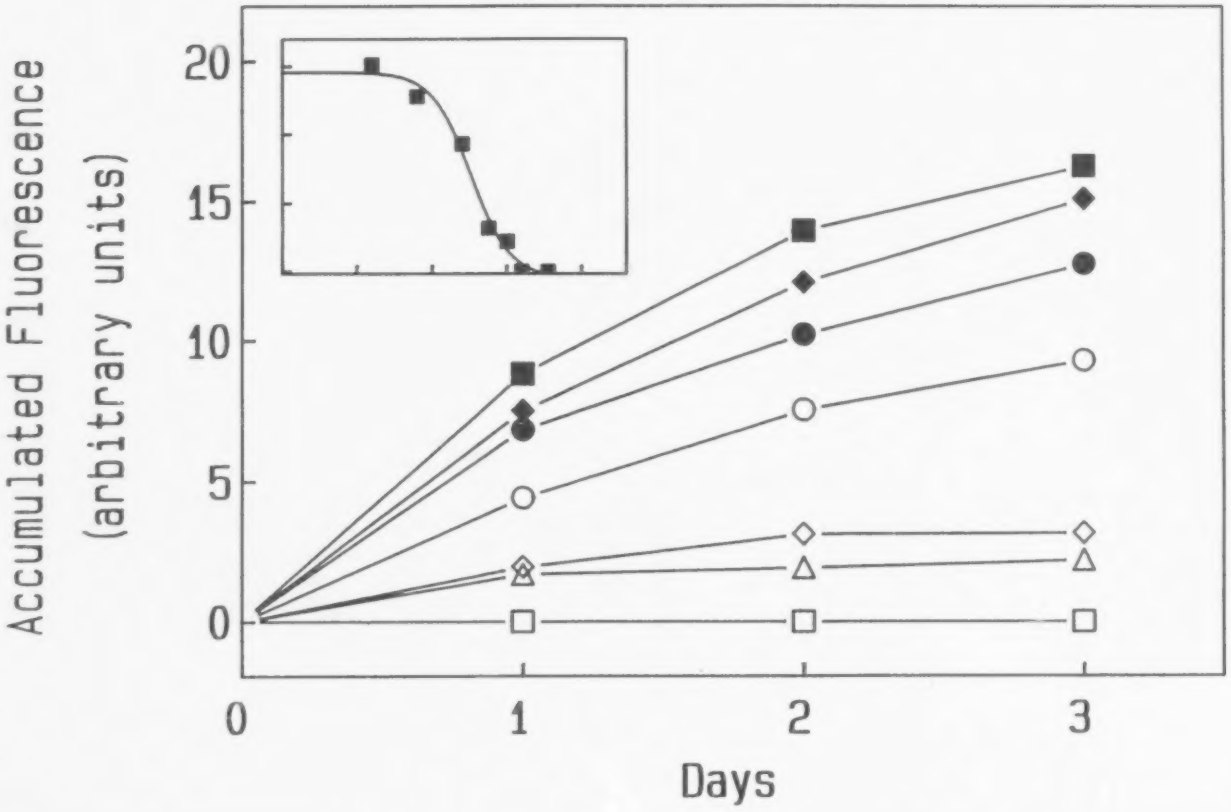


Figure 34. Effect of varying concentrations of the synthetic metalloproteinase inhibitor Z-Pro-Leu-Gly-NHOH on the digestion of FITC-collagen by human gingival fibroblast (strain HUGE 15). Inhibitor concentrations tested were: ■ 0, ◆ 20 μM, ● 40 μM, ○ 80 μM, ◇ 120 μM, △ 160 μM, □ 200 μM. The inset shows a plot of the activity at 3 days versus Log_{10} [inhibitor concentration] for the same experiment. The IC_{50} calculated from these data was 91.7 μM. Results are average for triplicate wells.

IV. DISCUSSION

A. Purification of Matrix Metalloproteinases

It was essential to purify telopeptidase and use it to determine the scissile bond since it was intended to base the inhibitors to be synthesized on the amino acid sequence around this bond. Porcine gingival explant medium was used in the previous work done in this laboratory (Scott and Goldberg; 1983) since the rate of turnover of collagen in gingiva is known to be high (Sodek, 1977). Enzymes found in culture medium conditioned by gingival explants could conceivably originate from any of the cell types present. This includes fibroblasts, epithelial cells, mast cells, macrophages and leukocytes of various types. While care was taken not to include any inflamed tissue, the presence of some leukocyte elastase and collagenase cannot be completely discounted. In the present study cultured human gingival fibroblast medium was used for the purification of telopeptidase. These fibroblasts had been stimulated to secrete proteinases by concanavalin A, as suggested by Hurum et al. (1982). In the absence of such stimulation, the mechanism of which is poorly understood (Wang et al. 1983), little activity was found. Heath et al. (1982) were not able to detect any neutral protease production by human gingival fibroblasts, although these cells did secrete a potent inhibitor. The fact that no method of stimulating enzyme production was used may account for their results. An alternative explanation would be that different cell lines of gingival fibroblasts vary widely in their capacity to secrete proteinases (P.G. Scott,

C.M. Dodd, and J. Chai, unpublished data). The culture medium could not be very highly concentrated (1 litre was concentrated to 60 ml) due to the tendency of the matrix metalloproteinases to form oligomers or to aggregate. Activation of the conditioned and concentrated culture medium with trypsin-agarose resulted in increases in telopeptidase, collagenase and gelatinase activity, indicating that all three enzymes are present in latent forms. Trypsin cleaves between the Arg-55 and Asn-56 peptide bond of collagenase, generating a 46 kDa intermediate which is subsequently converted into a 42 kDa active enzyme (Grant et al., 1987). Trypsin removes 84 amino acids from the amino terminal end of prostromelysin (Wilhelm et al. 1987). It has been suggested by Collier et al.(1989) that progelatinase is a poor substrate for trypsin activation since it does not have the conserved KRR or RRK sequence present in the propeptides of collagenase and stromelysin. Cleavage with trypsin occurs on the C-terminal side of the peptide bonds of arginine and lysine. The bond Lys-Pro is not cleaved but, that of Arg-Pro can be cleaved (Kamp 1986). These workers did not investigate whether susceptible sequences are present in proMMP-2, but are inaccessible to trypsin. In our experience gelatinase in the crude culture medium was activated by trypsin but no study was done in order to determine if the activation was due to trypsin directly, to autoactivation, or as a result of other proteolytic activities present in the crude medium. During the purification steps there was a loss of protein. Recovery decreased with the number of steps involved although the specific activity of telopeptidase increased, hence it was important to keep the number of purification

steps to a minimum. Losses could be attributed to handling, concentration, assay and non-specific adsorption. The telopeptidase assay was done at temperatures (18°C to 25°C) which are well below physiological values. During the purification procedure and in experiments involving the use of explant medium, collagenase and other nonspecific proteinases are present. At physiological temperatures collagenase cleaves triple helical collagen, the fragments denature and are acted upon by gelatinases. Following such activity, it would not have been possible to demonstrate selective cleavage in the telopeptides.

After the gel filtration step the separation of the three enzymes of interest was not significant. The major telopeptidase-activity-containing fractions that were pooled for further purification contained some gelatinase and collagenase activity. Gelatinase elutes from Bio-Gel A-0.5m earlier than the rest of the activities and as a broad peak extending from a position corresponding to M_r 205,000. Earlier work done in this department (Nakano & Scott; 1986) has shown this gelatinase actually to be 72,000 kDa. It was suggested that the large apparent molecular weight seen at the initial step was due to the tendency to form oligomers or to aggregate with other components in the medium. This effect was only seen in medium that had been frozen for storage prior to concentration.

The lack of correspondence between the major peak of Azocoll-digesting activity and gelatinase on gel filtration was unexpected since Azocoll is essentially denatured collagen (gelatin), albeit in an insoluble particulate form, coupled to an azo- dye. Instead, Azocoll-digesting activity eluted with collagenase (apparent M_r

35 000). This activity may be due to other metalloproteinases (Barrett & McDonald, 1980).

On chromatofocusing collagenase did not bind to the column and hence eluted well separated from the other enzymes. This fraction was therefore pooled and used as purified collagenase. The collagenase activity in these fractions was very strong (2μl of the fraction digested 5 mm of the collagen gel in a capillary in less than two hours) and had to be diluted to use for assays during inhibition studies. Conditions during chromatofocusing should be chosen so that the protein of interest elutes between 3 and 5 bed volumes from the start of the gradient. Due to its basic nature (Woolley et al. 1975) collagenase elutes immediately on chromatofocusing and was used as purified enzyme. Woolley et al. (1975) used QAE-Sephadex A-50 effectively to purify rheumatoid synovial collagenase since 95% of the total protein sample was retarded on the column while collagenase failed to bind. Stricklin et al. (1977) have shown that human skin collagenase absorbs to cation-exchange material, phosphocellulose, in 0.05 M Tris-HCl, pH 7.5 and was eluted after the major protein peaks in an $(NH_4)_2SO_4$ gradient. This fraction was also used to determine the K_m of collagenase. Using the Cornish-Bowden plot the $K_m$was found to be 1.12×10^{-6} M. Turto et al, (1977) determined the K_m for human leukocyte collagenase. Using soluble ^{14}Clabelled type I collagen and the Lineweaver-Burk plot the K_m was 1.04×10^{-6} M. The apparent isoelectric point of gelatinase was 4.15 (Nakano & Scott; 1986) which was close to that for rabbit uterus gelatinase reported to be 4.42 (Sakyo et al. 1983). There was weak

gelatinase activity associated with Fraction 1, which we believe is due to the gelatinolytic activity of collagenase itself (Welgus et al., 1985). Immediately after elution there was no significant telopeptidase activity associated with this fraction, but when checked after a few days there was measurable telopeptidase activity. Some of this fraction 1 was rerun on chromatofocusing (not shown) in an attempt to separate the two activities without much success. Sample was also passed on gel filtration (G-100) and on elution the collagenase-containing fractions did show telopeptidase activity (not shown). The reason for the increase in activity after three to four days is not clear, but as was found later on, using synthetic substrates, collagenase itself does have weak telopeptide-cleaving activity. Several investigators have suggested the presence of an enzyme-inhibitor, TIMP (Tissue Inhibitor of Matrix Metalloproteinase) complex with a stoichiometry of 1:1. Such a complex is unlikely in this study since trypsin was used to activate the culture medium and TIMP has been reported to be inactivated by proteolysis (Vater et al. 1979b). The presence of some other slowly-dissociating inhibitor cannot be discounted. Clark and Cawston (1989) have shown that active human fibroblast collagenase on storage autolyses to produce a small (22 kDa) fragment that retains the active site and is capable of activating procollagenase. They suggested that the smaller size of the active fragment may enhance it's ability to penetrate the extracellular matrix and gain access to the substrate. The possibility that this 22 kDa peptide is responsible for the telopeptidase activity in fraction 1 off chromatofocusing cannot be dismissed. Telopeptidase activity eluted in two

different fractions on chromatofocusing. These were pooled and processed separately on Phenyl-Sepharose. Hydrophobic interaction chromatography on Phenyl-Sepharose without using ammonium sulphate (ie the second Phenyl-Sepharose) seemed to be an essential step in the separation of telopeptidase and gelatinase. Gelatinase due to its hydrophobicity (Collier et al; 1988) binds very strongly on the Phenyl-Sepharose column. Telopeptidase 2 (Fraction 3) after chromatofocusing did not show any collagenase activity, confirming that it was indeed a different enzyme, separable from collagenase. The CNBr digestion study using Fractions 2 and 3 (telopeptidases 1 and 2) was done within a week of the final purification step. The determination of the scissile bond(s) for telopeptidase 2 on the synthetic peptides (see later) was also done soon after purification. Telopeptidase 1 was not tested until several months later, by which time most of its activity had been lost and it did not show measurable activity towards any of the synthetic peptides. This enzyme was probably not stable under the storage conditions used. Both purified telopeptidases 1 and 2 showed PDS-digesting activity. From previous work Nakano & Scott (1988) showed the presence of two PDS-digesting activities in conditioned fibroblast medium, one with an acidic pH optimum and another with a neutral pH optimum. The pH optimum study was done on telopeptidase 2 in order to check if it might be this particular acidic metalloproteinase but its pH optimum was found to be close to neutral. The reason for the complexity of the elution profiles of the three MMPs at the different steps of purification is not known with certainty but may be related to the

occurrence of complexes of enzyme and other components (see later).

B. Large Scale Incubations of Telopeptidase 1 and 2 with Collagen

The C-terminal extra-helical region of collagen is more susceptible to proteolytic digestion than the N-terminal telopeptide, during the extraction and purification of collagen from the tissues (Stoltz et al., 1972,1973). In this study acetic acid soluble collagen was isolated in the presence of several proteinase inhibitors. Even so not all of the collagen had an intact C-terminal telopeptide (approximately 30-40 % of the α_1CB6 was in the b and c forms). The digestion of collagen by telopeptidase 1, when examined on 5% SDS polyacrylamide gels, showed significant collagenase activity, while telopeptidase 2 showed no such activity. It was not possible to determine if the collagenase activity in telopeptidase 1 was actually associated with telopeptidase *per se*, or if it was due to poor resolution of collagenase on chromatofocusing. Fraction 2 from chromatofocusing was very broad.

Incubation of telopeptidase 1 and 2 with the collagen substrates with subsequent CNBr digestion, and separation on 12 % Laemmli gels showed significant differences in the α_1CB6 region. Both the fractions caused a decrease in the α_1CB6a with telopeptidase 1 showing an equivalent increase in the α_1CB6c peak, indicating that most of the collagen C-telopeptide was lost, and the telopeptidase 2 digest showing an equivalent increase in the α_1CB6b band, indicating that only part of the collagen C-telopeptide was lost. The telopeptidase 1 digest also showed a product of collagenase activity (α_1CB7') which runs very

close to and sometimes overlaps the α_1CB6a band. Thus there are at least 2 different telopeptide-cleaving activities present in the gingival fibroblast culture medium. It was not possible to establish whether telopeptidase 1 activity was actually due to collagenase (see later) or to a separate enzyme.

C. Collagenase as a Telopeptidase

Cawston and Tyler (1979) purified collagenase using gel filtration, DEAE-cellulose, heparin-Sepharose and zinc-chelate-Sepharose chromatography. They indicated their collagenase preparation to be at least 94% pure. A sample of this preparation (kindly supplied by T.E. Cawston), was demonstrated to contain a significant level of telopeptidase activity (Goldberg, 1985). In the present study telopeptidase 1 (Fraction 2 off chromatofocussing) showed the presence of collagenase activity. This, in addition to the previous efforts where it was found virtually impossible to separate the collagenase and telopeptidase activities, raised the question of whether collagenase itself might have telopeptide-digesting activity. To investigate this possibility purified collagenase (Fraction 1 off chromatofocusing) was tested against the synthetic peptide substrate Nα_1C-telo and found to cleave at the Ser-Phe peptide bond. One could argue that some telopeptidase had eluted in this fraction during chromatofocusing. Human recombinant collagenase was also tested against the two synthetic telopeptides and found to cleave both the peptides at other sites in addition to those cleaved by telopeptidase 2. The α_1C-telo-2 peptide was cleaved at the Lys-Ala peptide bond and the Nα_1C-telo peptide

was cleaved at the Asp-Leu and Ser-Phe peptide bonds. Hence in our purification, the telopeptidase activity seen in Fraction 1 (collagenase) could not be due to contamination by telopeptidase 2. This is the first study showing collagenase to be capable of cleaving the nonhelical C-telopeptide region of type I collagen. Collagenase is known to cleave gelatin at Gly-Leu, Gly-Ile, Gly-Phe, Gly-Ala and Gly-Val peptide bonds with cleavage preferences in that respective order, with the Gly-Leu cleavage seen in less than 20 min incubations and Gly-Ala and Gly-Val cleavage seen only after several hours (Welgus et al.1985). Fields et al (1990) have shown that human fibroblast collagenase has a single active site that is capable of hydrolysing a much wider variety of natural and synthetic substrates then previously believed, such as collagen, gelatin, casein and collagenase (autoactivation). The cleavage which we found at the Lys-Ala peptide bond could not be responsible for the telopeptidase activity in the assay, nor could it be responsible for the initial breakdown of fibrillar collagen, since it is on the C-terminal side of the Lys residue involved in cross-linking. The cleavages between the Asp-Leu and Ser-Phe peptide bonds by collagenase are relatively weaker when compared to the cleavages by telopeptidase 2, nevertheless, this activity could account for the reported ability of highly purified collagenase to slowly degrade fibrillar collagen (Vater et al., 1979a). Investigations from this laboratory have demonstrated that only about one in five C-telopeptides need to be cleaved for the release of collagen monomers from the fibrils (Goldberg & Scott., 1986). Although human fibroblast collagenase can now be acknowledged to hydrolyze substrates

other than just interstitial collagens, it should be underscored that it is still its activity towards these collagens that distinguishes it from other enzymes. The basis for this remarkable selectivity is not fully understood, but it not due to the sequence specificities of these enzymes. This ability is reflected in the low K_m values for hydrolysis of collagen (1 μM) compared to peptides and other protein substrates (Fields et al 1987). Highberger et al.(1979) put forth a hypothesis, that there are "locally unstable" regions of the triple helix brought about by a local defficiency of imino acids. The presence of a cleavable sequence in an unstable region could expose the scissile bond to the enzyme and account for the observed specificity. It is possible that the enzyme(s) that are synthesized by cells have one primary function but may have one or more secondary functions in the breakdown of extracellular matrix.

D. Determination of the scissile bonds for Telopeptidase 2 and Stromelysin

The rational design and synthesis of active site-directed inhibitors of telopeptidase would be possible knowing the scissile bond(s) within the C-telopeptide. From the electrophoretic mobility of the shortened form of α_1CB6 produced by incubation of soluble collagen with the purified telopeptidase 2 it was already known that this enzyme catalyzes hydrolysis close to and on the N-terminal side of the lysine residue which is involved in intermolecular cross-linking. Previous efforts to isolate the small peptides released from the soluble collagen and to characterize them by amino acid composition were unsuccessful due to insufficient

material for an unambiguous result. Thus it was decided to use synthetic peptides corresponding to the C-telopeptide and purification of the fragments produced on HPLC in order to determine the scissile bond(s).

The digest of the synthetic peptide corresponding to the entire C-telopeptide of collagen with the concentrated active explant medium contained several unidentifiable fragments, mainly due to the presence of several activities in the explant medium. Further problems arose because this substrate was contaminated with several deletion peptides, including ones missing the N-terminal G, GP or GPP sequence and/or the C-terminal Y residue, and hence elutes on RP-HPLC as a broad peak. On cleavage even by purified telopeptidase a complex pattern was observed. Thus it was essential to synthesize smaller substrates. PQPPQE is an unique sequence present only in the C-terminal non-helical region of the α_1 chain of type I collagen and hence was included in the α_1C-telo-2 sequence: Ac-PQPPQEKAHDGGRYY-NH_2. Tyrosines were included to facilitate detection of the cleavage products on HPLC by monitoring at 280 nm. In spite of their quite different sequences, the two cleavage products of this peptide produced by telopeptidase 2 coeluted on RP-HPLC when the TFA/H_2O - TFA/CH_3CN system was used. Hence other purification methods had to be attempted. Cation exchange HPLC seemed a logical next step. This method requires desalting each peak on RP-HPLC, and hence is tedious on a routine basis and losses increase with the number of steps involved. After RP-HPLC one of the peaks was identified as KAHDGGRYY. The other peptide (PQPPQE) was lost during the desalting step,

probably due to the small amount of sample. The low uv absorbance of PQPPQE would also make it very difficult to detect. Based on the property of HFBA to retain positively charged peptides longer on RP-HPLC, it was found possible to separate two cleavage products of telopeptidase 2 action on α_1C-telo-2.

The sequence including the N-terminal sequence of the C-telopeptide of the α_1 chain of type I collagen was found by Scott & Pearson (1981) to be cleaved by cathepsin D at the Leu-Ser peptide bond. Other minor cleavages, at the Ser-Phe and either or both of the Asp-Leu and Phe-Leu peptide bonds were detected by amino terminal determination. Cathepsin D is able to solubilize fibrillar type I collagen, (Scott & Pearson, 1978b). This hydrophobic region of the C-telopeptide of type I collagen seems to be quite susceptible to proteolysis. Therefore the peptide Ac-GPPSGGYDLSFL-NH_2 was synthesized separately and found to be cleaved by telopeptidase 2 and purified MMP-3 at two sites. The cleavage between Asp and Leu was the major one and that between Leu and Ser minor. One of the fragments, -SFL-NH_2, was not detected. The other fragment Ac-GPPSGGYDL- resulting from this cleavage was found by amino acid analysis. Recombinant MMP-3, however, did not show the minor cleavage (at the Leu-Ser peptide bond). Hence the possibility that the minor cleavage by telopeptidase and purified MMP-3, was due to another contaminating proteinase cannot be ruled out. The -Asp-Leu- sequence in aggrecan was also found to be cleaved by PUMP (Fosang et al. 1992), the rat analog of stromelysin.

E. Identity of Telopeptidase 2 and Stromelysin / MMP-3

In the earlier studies it was difficult to assess the relationship between telopeptidase and connective tissue metalloproteinases other than collagenase and gelatinase since other workers had not tested their enzymes for activity against the C-telopeptides of collagen. The CNBr peptides of soluble collagen incubated with either telopeptidase 2 or stromelysin showed an increase in the αCB6b band, suggesting that they are both capable of cleaving the C-telopeptide between the helix and the cross-link. Several researchers have shown that stromelysin is capable of cleaving a variety of extracellular matrix substrates such as proteoglycans, fibronectin, laminin, gelatins and collagen types III, IV, V, IX (Matrisian, L.M. 1992 and references therein; Review). However, this is the first report showing that stromelysin cleaves the C-telopeptide of type I collagen. The study done with the synthetic peptides using telopeptidase 2 and recombinant stromelysin also shows similar results, that is both cleave between Glu and Lys (major cleavage) and between Asp and Leu (minor cleavage). Telopeptidase 2 also cleaves fibronectin, proteodermatan sulphate and gelatin of type I collagen. Gunja-Smith et al (1989) made a comparison of purified neutral proteoglycan-degrading cartilage metalloproteinase with authentic MMP-3 from human rheumatoid synovial cells, using various protein substrates. They showed that the digestion patterns of casein, carboxymethyltransferrin, gelatin of type I collagen and fibronectin were identical for the two proteinases. They concluded that the purified neutral metalloproteinase of cartilage was identical with human MMP-3

from synovial fibroblasts. A comparative study similar to theirs was not possible due to lack of sufficient telopeptidase 2. The cleavage sites of purified and recombinant stromelysin on the synthetic C-telopeptides of type I collagen were identical to those of telopeptidase 2. Hence based on the knowledge of the specificities of these enzymes we were able to conclude that telopeptidase 2 was identical to stromelysin / MMP-3.

F. Fibrillar Collagen Breakdown

There is disagreement in the literature concerning the actual role of collagenase in the turnover of fibrillar collagen. Collagen molecules in the fibrils are axially displaced by an integral multiple of D (670nm) (Bornstein & Traub, 1979). The lateral (azimuthal) arrangement of the molecules within the fibrils (50-100 nm in diameter) has not been established. The formation of fibrils depends on the strong electrostatic and hydrophobic interactions between the collagen molecules. Intermolecular cross-linking stabilizes this arrangement. Welgus et al. (1980) have demonstrated *in vitro* that only collagen molecules on the fibril surface are immediately accessible to collagenase. Only after removal of these outer layers of collagen molecules could the collagenase act on the underlying substrate. While the gap region (the area between two collagen molecules lying end to end) is near to the collagenase cleavage site, the C-terminal end of the helix and the C-terminal telopeptide of one monomer in fact overlie the cleavage site (Fig 35) in the underlying molecule and thus may act as a physical barrier to collagenase. Most

researchers use reconstituted collagen as substrate. The rate of degradation of such collagen is less than $1/10^{th}$ of that of collagen in solution. Vater et al., (1979a) introduced cross-links into ^{14}C-labelled lathyritic collagen and showed that an average of 0.59 Schiff base cross-links per collagen molecule almost completely prevented digestion by purified collagenase. An increase in the enzyme concentration and/or time of incubation did eventually solubilize the collagen. It was suggested that the ^{14}C-labelled substrate did not have a sufficient number of intermolecular cross-links to covalently link all the collagen molecules. Woolley et al. (1978) have shown that the rate of degradation of polymeric collagen (prepared by 'α-amylase dispersion') by collagenase was only 5% of that of non cross-linked reconstituted collagen. Based on our work with recombinant collagenase and synthetic C-telopeptides of collagen we believe that the weak activity of purified collagenase on the lathyritic collagen in Vater's experiments or on polymeric collagen in Woolley's work could possibly be due to the weak telopeptidase activity of collagenase.

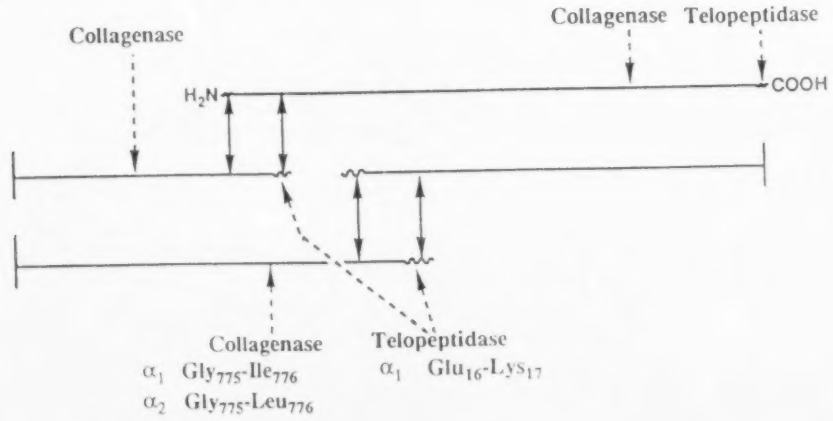


Fig 35. A schematic representation of the location of the major sites on collagen that are cleaved by collagenase and telopeptidase

G. Other Factors that may Modulate Collagen Turnover

There may be other factors which modulate collagenase activity *in vivo*. Certain other extracellular components such as proteodermatan sulphate and fibronectin have been demonstrated to interact with collagen. Scott, J.E. (1980) presented electron microscopic evidence for an interaction of proteodermatan sulphate with fibrillar collagen (using a stain for sulphated glycosaminoglycans). The proteoglycan appeared to be associated with collagen at regular intervals along the fibril in areas that correspond to the 'gap region'. An interaction of this nature might possibly impede entry of proteinases such as collagenase into underlying parts of the fibril. Fibronectin has also been shown to interact with collagen, in *in vitro* experiments, specifically in the region containing the sequences susceptible to collagenase (Kleinman et al., 1981). In addition, fibronectin has been shown to affect the rate of degradation of different types of collagen by collagenase, presumably for the reason described above (Menzel & Borth, 1983). Differences in the rate of digestion of different collagen types were related to the relative affinities of the collagen types for fibronectin. There are several studies which have demonstrated proteoglycan-degrading activities in cultures of connective tissues (e.g. Galloway et al, 1983; Nakano & Scott 1988). Previous work in this department has shown that acetic acid soluble collagen may contain some proteodermatan sulphate (or at least the protein core). In our study both telopeptidase 1 and 2 were found to be capable of cleaving proteoglycan. Thus

it is possible that in addition to the cleavage of the C-telopeptide, removal of matrix molecules such as fibronectin and proteoglycans may be important for the degradation of fibrillar collagen.

H. Inhibition Studies

There have been several reports on synthetic inhibitors of vertebrate collagenase. Most of these contain a metal chelating group replacing the carbonyl group present at the scissile bond in the substrate, and a short peptide sequence similar to that in the substrate [Bartlett & Marlowe(1983), Delaisse et al.(1985), Gray et al.(1981), Kortylewicz & Galardy (1990), Mookhtiar et al.(1987), Moore & Spilburg (1986), Okade et al.(1990)]. MMP-3 has been implicated in diseases such as rheumatoid arthritis (Okada et al., 1989b) and osteoarthritis (Martel-Pelletier et. al. 1984; Dean et al., 1989). The early depletion of proteoglycans from cartilage coincides with the appearance of stromelysin in arthritic joints. Both stromelysin and collagenase are evident within 48 hrs of the onset of inflammation induced in animal models (Hasty et al. 1990). MMP-2 is presumably required for the turnover of basement membrane in vivo, but increased secretion has also been shown to be linked with the invasive potential of tumor cells (Liotta, et al.1986., Tryggvason et al. 1987., Zucker et al., 1990). The latter may significantly facilitate the penetration of disseminating tumor cells through tissue compartments of the body. Thus inhibitors to MMP-2 would be of significant value. Highly selective inhibitors to all three enzymes could be of great therapeutic use for control of the

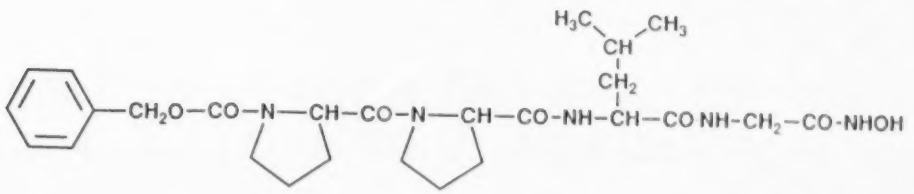
tissue destruction associated with various diseases.

The design of inhibitors to be synthesized in this study was based on the idea that substrate analogs would bind strongly at the active site and a zinc chelating group attached to the peptide would inactivate the enzyme. To make such inhibitors it is essential to know the scissile bond of the enzyme, or to know the structure of the active site of the enzyme. Several research groups are now trying to obtain a crystal structure of a representative member of the MMP family (Lloyd et al.; 1989), but none has so far been published. Thermolysin (*Bacillus thermolyticus*), a zinc metalloproteinase which has been studied extensively by X-ray crystallography is used by most investigators as a model for matrix metalloproteinases. Its crystal structure with an inhibitor has been determined (Matthews; 1988). Zincov [2-(N-hydroxy-carboxamido)-4-methylpentanoyl-L-alanyl-glycine amide], a commercially available inhibitor of thermolysin, was found to be a weak inhibitor of partially purified telopeptidase from gingival explant culture medium (25% inhibition at a concentration of 0.3 mM). HONH-Bzm-L-Ala-Gly-NH_2 (compound #10), another good inhibitor of thermolysin with an N-terminal hydroxamic acid to chelate the active-site zinc, was synthesized primarily to test the feasibility of this approach but nevertheless was expected to be an inhibitor of gelatinase because of its structural similarity to Gly-L-Phe-L-Ala-Gly-, one of the sequences within gelatin known to be degraded by skin gelatinase (Seltzer et al. 1981). However the results were disappointing when compared to the C-terminal peptide hydroxamates (Z-Leu-Gly-NHOH and Z-Pro-Leu-Gly-NHOH) that were

synthesized and tested at the same time. Compound #10 inhibited gelatinase poorly (only 35% inhibition at 1mM concentration). At 1mM concentration only 36% of collagenase and 15% of telopeptidase activity was inhibited by compound #10. Therefore, because of the lengthy and difficult synthesis involved and the weak inhibition obtained, the approach of making N-terminal peptide hydroxamates was dropped. Recently Grobelny et al. (1992) have used the N-terminal hydroxamate approach (see later).

In our study, several di-, tri-, and tetra Z-peptide-hydroxamates were synthesized and tested on the purified enzymes, most extensively on gelatinase, due to the simplicity of the assay requiring small amounts of inhibitor and short incubation times. Also after testing a few of the inhibitors synthesized, it was found that all were much better inhibitors of gelatinase than of either of the other activities. All three enzymes followed a similar trend of inhibition by these hydroxamates. The degree of inhibition increased with the length of the peptide and inhibition by all the inhibitors was most effective for gelatinase and weakest for collagenase. Collagenase has a triple helical substrate making it very difficult to synthesize an inhibitor that is a true substrate analog. For collagenase a chemical structure that would represent the surface structure of triple helical collagen would probably be best. The K_i values were not determined for all inhibitors since several concentrations of the substrate are required and at high concentrations solutions of gelatin (for gelatinase) or collagen (for collagenase and telopeptidase) tend to be very viscous, making kinetic studies difficult. Collagen, the natural substrate of

collagenase, is a triple helical molecule. The inhibitor Z-(D)-Pro-Pro-Leu-Gly-NHOH was synthesized because on computer models the distance between the Leu and Z residues appeared to closely match that of the two hydrophobic residues on the two α chains of collagen that lie side by side (Scott, P.G., unpublished results). However it was found to be a slightly weaker inhibitor than Z-(L)-Pro-Pro-Leu-Gly-NHOH, suggesting that the inhibitor with (D)-Pro at P_4 was not a better fit to the enzyme's active site.



Z-Pro-Pro-Leu-Gly-NHOH

In later studies, in order to save time and reduce losses in yield during the conversion of peptide acid into hydroxamate, it was decided to synthesize a number of peptides as Z-peptide-acids or Z-peptide-amides and to screen these for inhibitory activity. The idea was that the best one could then be made into an hydroxamate. The acids Z-Pro-Pro-Gly-COOH, Z-Pro-Pro-Leu-Gly-COOH and Z-Gly-Pro-Pro-Gly-COOH were initially synthesized but inibition was better with the amides. Perhaps the charge on the C-terminal carboxylic group of acids interferes with binding. Hence peptide amides with four amino acids were synthesized.

Amino acids at position P_3 and P_4 (both being prolines) were kept constant because the substrate for telopeptidase contains prolines at both these positions. To investigate the amino acid that would be best fit at the P_1 position, several peptides with the sequence Z-(L)-Pro-Pro-Leu-Xaa-NH_2 were synthesized, where "Xaa" was Lys (+charge), Asp or Glu (- charge), Leu (hydrophobic residue), Cys (amino acid with -SH group) or Gln. The peptide Z-(L)-Pro-Pro-Gln-Gly-NH_2 was synthesized with a Gln substituted for Leu at position P_2 because the C-telopeptide has a Gln at that position. Thus Z-(L)-Pro-Pro-Gln-Gly-NH_2 and Z-(L)-Pro-Pro-Leu-Gly-NH_2 can be compared (we knew Z-(L)-Pro-Pro-Leu-Gly-NHOH was a good inhibitor of MMP-3). Finally the peptide amide Z-(L)-Pro-Pro-Gln-Glu-NH_2, which would represent the exact sequence in the substrate on the N-terminal side of the cleavage site of MMP-3, was made.

Several problems were associated with the testing of these amides on telopeptidase 2 / MMP-3. Purified MMP-3 was used in the inhibition studies. This preparation had some contamination of collagenase. The assay that was used was not the assay specifically developed for telopeptide cleaving activity (using tritiated collagen substrate), instead carboxymethylated transferrin used by other researchers and kindly provided to us by Dr. Nagase was used. The inhibition of MMP-3 by all the amides was weak and high concentrations had to be used. The peptides were insoluble at concentrations higher than 6 mM. Z-Pro-Pro-Leu-Leu-NH_2 was insoluble even at lower concentrations and hence no data could be obtained. It was not possible to calculate the IC_{50} using the computer program,

GraphPAD InPlot™ for MMP-3 because a wide range of data, which is necessary to get a sigmoid plot, was not available. Z-Pro-Pro-Gln-Glu-NH_2 showed no significant inhibition of MMP-3 and Z-Pro-Pro-Leu-Gly-NH_2 inhibited its activity only by 14% at 5 mM concentration. It can thus be concluded that Z-Pro-Pro-Leu-Gly-NH_2 is a better inhibitor of MMP-3 than Z-Pro-Pro-Gln-Gly-NH_2, suggesting that although Gln is present at this position (S_2) in the substrate, Leu is probably a better fit in the enzyme's active site. The inhibitor Z-Pro-Pro-Gln-Glu-NH_2 includes the four amino acids N-terminal to the cleavage site of MMP-3 in the C-telopeptide of collagen but was also a weaker inhibitor than Z-Pro-Pro-Leu-Gly-NH_2, suggesting that making Z-Pro-Pro-Gln-Glu-NHOH may not be worthwhile as it is very unlikely to show better inhibition towards MMP-3 than Z-Pro-Pro-Leu-Gly-NHOH. The inhibition of gelatinase by these amides was better.

At position P_1 the presence of an acidic, basic or a neutral residue did not make significant difference in the inhibition of MMP-2, although a hydrophobic residue (Leu) as compared to a Gly at this position decreases the inhibition. The IC_{50} for Z-Pro-Pro-Gln-Glu-NH_2 was 5.59 mM as compared to 1.1 mM for Z-Pro-Pro-Leu-Gly-NH_2. Z-Pro-Pro-Leu-Asp-NH_2 showed the strongest inhibition of MMP-2 (IC_{50} of 0.7 mM) among the amides tested. Z-Pro-Pro-Leu-Cys-NH_2 has a thiol group on the side chain of cysteine and since thiols have been reported as inhibitors of MMPs (see later), better inhibition of the enzymes was expected, but it inhibited MMP-2 only weakly. Based on the inhibition results of the peptide amides, in the present study, it is not possible to conclude that they bind at the

active site.

The sequence -Glu-Lys- is adjacent to the cross link and may not be accessible to the enzyme in fibrillar collagen, however the cleavages at the -Asp-Leu- and -Leu-Ser- peptide bonds could be better accessible and hence important in the initial solubilization. It would be useful to have inhibitors that mimic the peptide sequences around the Asp-Leu and Leu-Ser scissile bonds. Scott & Pearson; (1981) have shown that cathepsin D, which is capable of solubilizing fibrillar collagen, cleaves the C-telopeptide of type I collagen closer to the helix (between the Leu-Ser peptide bond).

Recently Enghild, et al. (1989) have shown that human fibroblast collagenase cleaves α_2-macroglobulin at the Gly-Leu peptide bond in the small collagen-like bait region sequence -Gly-Pro-Glu-Gly-Leu-Arg-Val-Gly-. Alpha$_2$-macroglobulin is a 150-fold better substrate for collagenase than is type I collagen (Sottrup-Jensen, and Birkedal-Hansen, H. 1989). They suggest that the triple helical structure may not be an absolute requirement for the binding of collagenase to its substrate. Peptide inhibitors including the amino acid sequences before and after the peptide bond cleaved by collagenase in the bait region of α_2-macroglobulin might be worth testing.

In the past few years research on inhibitors of matrix metalloproteinases (MMP-1 in particular) has advanced a great deal. Recent findings can be summarized:

Tetracyclines, particularly minocycline, were found to inhibit the pathologically excessive mammalian collagenase activity in gingiva from diabetic rats and in

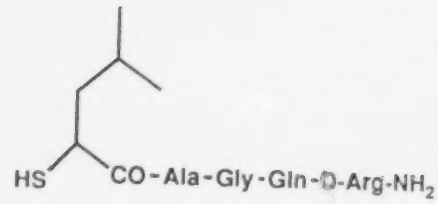
gingival fluid from humans with inflammatory periodontal disease (Golub et al. 1983; Golub et al. 1985). Partially purified collagenases from rat neutrophils or rabbit chondrocytes were inhibited by incubation with minocycline but not by non tetracycline antibiotics, and the mechanism was partially reversible by addition of excess calcium (Golub et al. 1983; Golub et al. 1985). The mechanism of this action does not depend on the antimicrobial activity of the drugs and is probably due to chelation of metal ions by the drugs and/or formation of an inhibitor/enzyme complex with decreased activity.

A number of thiols have been reported to inhibit collagenase at millimolar concentrations. These include cysteine, dithiothreitol and cysteine or penicillamine containing peptides. A number of naphthalene or benzothiazole containing compounds have been described, which prevent the degradation of collagen fibrils by human collagenase at concentrations between 10-100 micromolar. One example of this class of compounds is WY-45,368, N-[[(5-chloro-2-benzothiazolyl)thio]phenyl-acetyl]-L-cysteine (Clark et al., 1985) with an IC_{50} of 10 μM against human fibroblast collagenase using both Type I and Type II collagen as substrate. WY-45,368 does not inhibit thermolysin, angiotensin converting enzyme or clostridial collagenase.

Inhibitors Based on Substrate Structure

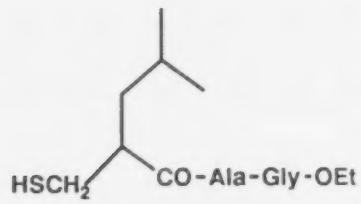
C-Terminal Sequences Containing Thiol Inhibitors

Gray et al. (1981) have shown that 2-mercapto-4-methylpentanoyl tetrapeptide (I) has an IC_{50} = 10μM against tadpole collagenase.



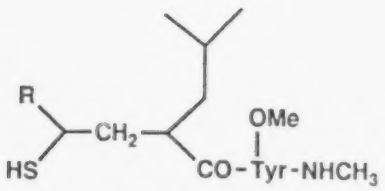
(I)

Thiol group and a methylene spacer between the thiol and the side chain were used to give compounds with activities below 10 μM (II) (Gray et al., 1986).



(II)

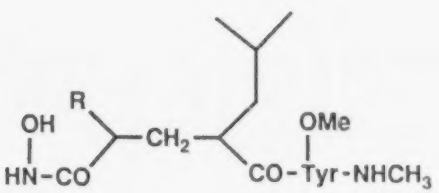
The length of the inhibitor did not markedly increase potency. The preference of collagenase for a leucine side chain adjacent to the cleavage site was confirmed, replacement of the isobutyl group by phenyl or methyl significantly reduced activity. A further improvement in activity has been achieved by the alkylation of the methylene spacer adjacent to the thiol ligand (Donald et al., 1985) In a series of acylated amino acid amides (III) the nature of the R group and the stereochemistry at both centres had a marked effect on the activity. The most active compounds (R = Me or Ph) had IC_{50} values of 2.2 and 2.7 x 10^{-7}M.



(III)

C-Terminal Sequences Containing Hydroxamates as Inhibitors

The hydroxylamine group which co-ordinates to zinc as a bidentate ligand, along with the C-terminal sequence, has been used successfully in inhibitors of the MMPs.

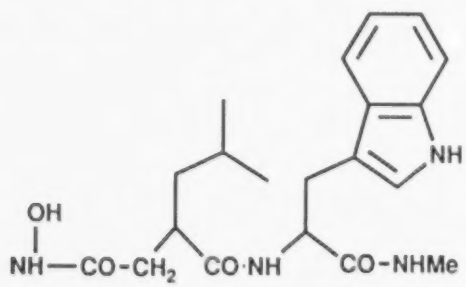


(IV)

In Compound IV synthesized by Dickens et al., (1986), when R =H, the K_i for the two diastereoisomers is 0.02 and 4.0 μM. In this series of compounds, the addition of an extra alkyl group (R = CH_3-) gave no improvement in activity. Removal of the isobutyl group or replacement by methyl gave compounds which were at least 10^4 times less active.

Grobelny et al., (1992) have synthesized diastereomers at the CH_2CH(i-Bu)CO α-carbon of the hydroxamic acid $HONHCOCH_2CH$(i-Bu)CO-L-Trp-NHMe (GM-6001 A). The diastereomer analogous to the L,L-dipeptide was found to be a selective inhibitor of skin fibroblast collagenase and inhibited the skin fibroblast collagenase

with a K_i of 0.4nM when assayed with a synthetic thioester substrate Ac-Pro-Leu-Gly-SCH(iBu)CO-Leu-GlyOEt. Its K_i was 50 times higher for thermolysin or P. *aeruginosa* elastase. The other diastereomer (GM-6001 B) analogous to the D,L-dipeptide, was a much weaker inhibitor of collagenase but inhibited the other two enzymes strongly. Thus they have shown that simple inversion of configuration at the CH_2CH(i-Bu)CO α-carbon atom of this inhibitor, allowed it to discriminate between human and bacterial enzymes. This is the most potent inhibitor of human skin fibroblast collagenase reported to date and is very selective for collagenase when compared to thermolysin, *P. aeruginosa elastase*, angiotensin-converting enzyme and plasmin.



GM-6001

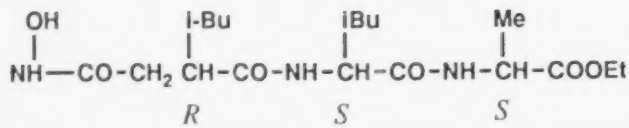
isomer-A(*L*) Ki = 0.4nM
isomer-B(*D*) Ki = 0.2μM

The requirement of collagenase for a leucine or isoleucine residue adjacent to the cleavage site is again demonstrated with these compounds. As summarized above, and emphasized by Grobelny et al., (1992), the S_1' subsite specificity for collagenase differs from that of thermolysin and *P. aeruginosa* elastase and this difference could be important in the design of inhibitors. Other hydroxamate containing peptides inhibited both the collagenase and stromelysin formed by IL-1 stimulation of chondrocytes (DiPasquale et al., 1986).

N-Terminal Sequence Containing Inhibitors

Few inhibitors based on the sequence N-terminal to the collagenase cleavage site have been reported. Starting from acetyl hydroxylamine, $CH_3CONH\text{-}OH$ (IC_{50} =40 mM) and adding sequence related groups, resulted in a peptide hydroxamate, Z-Pro-Leu-Gly-NHOH with an IC_{50} of 40 μM (Moore and Spilburg, 1986). Corresponding peptides with different C-terminal functional groups, such as carboxylate, aldehyde and amide, showed little or no inhibition. Acetyl dipeptide thiolacids (R-CO-SH) inhibited the collagenase released from interleukin-1 stimulated chondrocytes (DiPasquale et al., 1986).

Johnson et al. (1987) carried out a systematic study of ligands and subsite requirements for collagenase, based on the synthetic hexapeptide substrate Ac-Pro-Leu-Gly-Leu-Leu-Gly-OEt. At the N-terminal end of the substrate (Ac-Pro-Leu-Gly-Ligand) they used -NHOH as the ligand, whereas at the C-terminal end (Ligand-Leu-Leu-Gly-OEt) -SH, -CONHOH, -N(OH)CHO, $-PO(OH)_2$ were used as ligands. In both directions peptides based on Ac-Pro-Leu-Gly-Ligand-Leu-Leu-Gly-OEt were synthesized. Based on this study they concluded that either the N-terminal or the C-terminal end of the molecule binds to collagenase but not both. They also synthesized peptides with changes in the side chains in the four binding subsites P_1'-P_4' and peptides where the scissile amide bond was replaced. When all the optimum groups were incorporated into one structure, the very potent collagenase inhibitor (V) was obtained, $IC_{50} = 8.5 \times 10^{-9}M$ ($K_i = 5.0 \times 10^{-9}M$).



(V)

To date, the search for other ligands closer in geometry to the amide group, which would allow both ends of the molecule to bind, has met with limited success. Compounds containing replacements for the cleaved amide, for example -$CH(OH)CH_2$- and -$COCH_2$- were very weakly active but the ketomethyleneamino compound Z-Pro-Ala-$NHCH_2CO$-CH_2-Leu-Ala-Gly-OEt (K_i = 60 + 16 μM) (Wallace et al., 1986) and a series of phosphonamides (K_i = 14-78 μM), in which the carbonyl at the cleavage site is replaced by PO(OH), show reasonable activity (Mookhtiar et al.; 1987).

In the present study we looked at sequences only N-terminal to the cleavage site of telopeptidase but the specificity could lie on either side. So far no such systematic and rational approach has been taken for gelatinase/MMP-2 and stromelysin/telopeptidase/MMP-3. All the research in this field has compared inhibition of collagenase with metalloproteinases such thermolysin, elastase, and angiotensin converting enzyme, but so far no reported study compares the inhibition between the matrix metalloproteinases although such a study would be most logical. The preferred cleavage by MMP-2 is N-terminal to hydrophobic

amino acids and the collagenase inhibitors so far reported would probably inhibit it as well, although no such study has been reported. Stromelysin seems to have a broad sequence specificity, and a study based on just one sequence may not yield the best inhibitor.

Although the approach taken by us and the other workers in this field was to make peptide analogs of the substrate, in addition to the amino acid sequence, the tertiary structure of the enzyme at the substrate binding site would be a major determinant of the best fitting structure that should probably be used in the design of inhibitors which need not be peptides.

I. Inhibition Studies with FITC-Labelled Fibrillar Collagen

In the studies done with FITC-labelled collagen fibrils onto which fibroblast were seeded, 10% (v/v) serum did not completely inhibit the collagen digesting activity of fibroblasts, whereas Z-Pro-Leu-Gly-NH0H at a concentration of 200 μM almost completely inhibited their activity. When tested in a test tube against purified telopeptidase 2, human serum 2.5 % (v/v) was found to cause 93% inhibition of 0.6 units. Human serum contains the natural metalloproteinase inhibitors α_2-macroglobulin and TIMP. Purified α_2-macroglobulin and TIMP, both kindly provided by T.E. Cawston, inhibited telopeptidase only weakly. These observations suggest that high molecular weight inhibitors such as α_2-macroglobulin (M_r = 725,000) and TIMP (M_r = 20,000) which are present in the serum, are unable to penetrate between the surface of the fibroblasts and the

collagen fibrils where proteolysis and/or phagocytosis must occur. Among the inhibitors studied, Z-PPLG-NHOH was found to be the best inhibitor of all 3 enzymes when experiments were done in the test tube, but the fibrillar collagen breakdown by fibroblasts in cell culture was better inhibited by Z-PLG-NHOH. At the end of the experiments, the fibroblasts on the cell culture plates were briefly trypsinized and counted. The number of viable cells before and after the test were found to be the same. This suggested that the peptide hydroxamate inhibitors were not toxic to the cells.

Although there was significant inhibition of the fibrillar collagen breakdown by fibroblasts by Z-Pro-Leu-Gly-NHOH, the objective (to define a role for telopeptidase in the initial breakdown of fibrillar collagen), was not completely realized for several reasons. All three MMPs were inhibited by the inhibitors tested, whereas in order to do the above study, it was essential to have an inhibitor which was specific towards telopeptidase and not the other MMPs secreted by fibroblasts. Telopeptidase 2/MMP-3 shows a major cleavage at the Glu-Lys peptide bond and a minor cleavage at the Asp-Leu peptide bond in the C-telopeptide of collagen. The peptide Z-PPQE was found to be a weaker inhibitor of MMP-3 than Z-PPLG in the amide form. MMP-3 is known to cleave several different proteins (fibronectin, type IV collagen, α_2M, aggrecan etc.) at different peptide bonds. In aggrecan it cleaves the Asn_{341}-Phe_{342} peptide bond (Fosang et al. 1991). Interestingly this Asn_{341}-Phe_{342} peptide bond in aggrecan is cleaved by four MMPs (MMP-3, 72 KDa gelatinase, 95 KDa gelatinase and PUMP) (Fosang et al. 1992).

MMP-3 does not cleave the Asp_{441}-Leu_{442} peptide bond in aggrecan but cleaves the same sequence in the C-telopeptide of type I collagen. It is possible that access to this site in aggrecan is denied to MMP-3 due to glycosylation, probably at the closely located dipeptide (human residues 338-339), whereas PUMP which lacks the C-terminal hemopexin domain was shown to cleave this peptide bond. In the $\alpha_2$macroglobulin bait region MMP-3 cleaves at the Gly_{679}-Leu_{680} and the Phe_{684}-Tyr_{685} peptide bonds (Enghild et al, 1989). Nagase et al. (1990) have shown that the His_{82}-Phe_{83} bond and the Glu_{68}-Val_{69} bonds in proMMP-3 are cleaved by MMP-3, yet the sequence Glu_{28}-Lys_{29} was not cleaved. MMP-3 activates procollagenase by cleavage at the Gln_{80}-Phe_{81} peptide bond (Suzuki et al, 1990). Very little is known about what determines substrate specificity among the metalloproteinases but it seems evident that the structure around the enzymes active sites should be considered rather than the sequence around one scissile bond.

J. Conclusion

In this study the three matrix metalloproteinases (MMP-1, MMP-2, MMP-4) were obtained in the purified forms. The scissile bonds of MMP-4 (telopeptidase 2) and MMP-3 (purified and recombinant) in the C-telopeptide of type I collagen were determined using synthetic peptide substrates. Judged by the specificities of telopeptidase 2 (MMP-4) and stromelysin (MMP-3) it was concluded that the two matrix metalloproteinases were identical.

Using purified and recombinant collagenase it was possible to show that collagenase has weak telopeptidase activity.

Di-, tri- and tetra- peptide hydroxamate inhibitors were synthesized and tested against the three MMPs. All three enzymes were inhibited by these inhibitors. All the inhibitors showed better inhibition towards gelatinase. Inhibition improved with increase in length up to four amino acids with Z-PPLG-NHOH being the best inhibitor. The specificity between the three MMPs towards the inhibitors did not differ drastically.

Some of these inhibitors were shown to inhibit the breakdown of FITC labeled fibrillar collagen by human skin and gingival fibroblasts.

K. Possible Future Studies

Fibrillar Collagen Breakdown

Based on the present work on recombinant collagenase, we propose that collagenase can cleave the C-telopeptide of type I collagen. Although this activity of collagenase was weaker than the -Glu-Lys- peptide bond-cleaving activity of telopeptidase 2, it is possible that collagenase alone can, to some extent, solubilize fibrillar collagen. The rate of solubilization would probably be greater in the presence of telopeptidase 2/MMP-3 because it cleaves at three sites in the C-telopeptide. It has been shown that MMP-3 is involved in the activation of pro-collagenase. Thus it is possible that MMP-3 initially activates collagenase and then helps in solubilization by acting as a telopeptidase, effectively loosening the fibrillar

structure and facilitating the action of collagenase.

Thus it is possible that telopeptidase and collagenase activities work hand in hand in the solubilization of fibrillar collagen. Synergism between collagenase and other matrix metalloproteinases in the breakdown of fibrillar collagen has been proposed by a number of investigators. However experimental evidence appears to be limited to the leukocyte proteinases. Sopata et al. (1974) incubated (at 37°C) insoluble human skin polymeric collagen with either a leukocyte homogenate, a partially purified leukocyte collagenase or rheumatoid synovial fluid. Low levels of solubilization were obtained when these were incubated separately, as well as when the purified collagenase was incubated with the rheumatoid synovial fluid. The extent of digestion was increased 10-fold when both leukocyte homogenate and rheumatoid synovial fluid were incubated together. It would be of considerable interest to purify large amounts of telopeptidase 1 and study telopeptidase 1 and 2 separately and in combination with collagenase in the digestion of fibrillar collagen, in order to attempt to determine which, if any, of these three enzymes are involved in the initial breakdown of fibrillar collagen. Since it is possible to obtain recombinant MMPs, synergistic experiments could be done to show that recombinant MMP-1 can solubilize fibrillar collagen when used in sufficient amounts and that the rate of this solubilization increases by the presence of active recombinant MMP-3.

A Simple Assay for Telopeptidase

Most workers use short synthetic substrates such as Pz-Pro-Leu-Gly-Pro-D-Arg (Pz-peptide) instead of the natural substrates for assays. In our study Pz-peptide was shown to be cleaved by activated medium used for purification but the activity was too weak to be followed during chromatography. The assay that was developed for detecting telopeptidase activity uses isolated and purified acid soluble collagen which is treated with ^{3}H-sodium borohydride which specifically labels the lysine (at C_{17}) in the C-telopeptides. This assay is advantageous because it is specific to proteinases that are capable of cleaving in the C-terminal telopeptide of type I collagen. However, it is expensive, the incubation time is long and does not allow one to do initial rate studies. Furthermore, the molecular weight of collagen is very high and it has a tendency to aggregate even at relatively low concentrations, thus making it difficult to do kinetic studies.

Attempts were made to set up an assay using a synthetic peptide analogous to that of the substrate. A seven residue peptide (Z-PQEKAHD-NH_2) was cleaved in free solution by MMP-3 but when attached to CH-Sepharose 4B by the side chain of lysine no digestion could be detected. This peptide was not cleaved when bound to the resin due to steric reasons, the cleavage site being next to the lysine that was attached to the bulky resin. In vivo this lysine is cross-linked and access of telopeptidase / MMP-3 to this cleavage site may be difficult due to steric hindrance. In order to set up a sensitive assay it is important to use a substrate with a low K_m or a high k_{cat}/K_m. Even for the peptide in solution that was cleaved

by MMP-3, the concentration required was quite high. From the inhibitor studies, proline at position P_4 could be suggested to increase binding and hence inhibition. If the sequence of the substrate used were extended on the N-terminal side to include the Pro at position P_4, binding of this substrate might increase therefore one might have a sensitive assay. Besides, other methods that do not involve a bulky group at lysine can be used. The side chain of lysine can be acetylated to neutralize the charge and after incubation of this substrate with the enzyme the free amino group generated could be detected.

The other cleavage site (Asp-Leu) might better serve the purpose of solubilizing fibrillar collagen. An assay could be set up using this sequence as a substrate. The K_m of telopeptidase (using explant medium) on soluble type I collagen is 25μM, while the K_m of MMP-3 (human) on α_2-macroglobulin (human) is 0.010μM (Enghild,J.J. et.al. (1989). Thus it might be useful to make a peptide that included the sequence around the scissile bond for MMP-3 in the bait region of α_2-macroglobulin. Unfortunately, the bait region of α_2-M has cleavage sites for almost all the enzymes belonging to the four classes of proteinases, and unless care is taken not to include such sequences, specificity requirements might not be met using this approach.

Purification of metalloproteinases

In order to purify one of the MMPs from a pool of several (as in the fibroblast culture medium), affinity chromatography can be performed using a specific

inhibitor to the particular enzyme of interest. This would reduce losses due to the number of steps involved as seen in our study. The best inhibitor found in this study (Z-Pro-Pro-Leu-Gly-NH_2) cannot be used for such a purpose because it is not specific towards any one MMP and would bind all three MMPs.

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